BIOMARKER DISCOVERY AND FOOD ALLERGY PROFILING DISTINGUISHES DOCK8 DEFICIENCY FROM ATOPIC DERMATITIS.

Thesis submitted by

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This PhD is dedicated to,

My parents, Thundthil Varghese George and Mariamma George

&

My beloved family: Sunny Jacob, Dr. Sony T Jacob and Dr. Ronnie T Jacob

Abstract

Metabolomics is metabolites profiling in biological matrices and is a key tool for biomarker discovery. Over the last decade, metabolomics studies have identified several relevant biomarkers involved in complex clinical phenotypes using diverse biological systems. Most diseases result in signature metabolic profiles that reflect the sum of external and internal cellular activities. Metabolomics signature profiles are useful in clinical care, biomarker discovery for several common and rare human diseases, and in understanding disease mechanisms and response to therapy.

Hyper immunoglobulin E (IgE) syndrome [HIES] is characterized by significantly elevated serum IgE levels (>2000UL) and recurrent bacterial infections from infancy involving the skin and the lungs, chronic eczema and eosinophilia. Bi-allelic mutations in dedicator of cytokinesis 8 (DOCK8) are responsible for a form of autosomal recessive type of HIES, characterized by increased IgE levels, atopic dermatitis, food allergies, recurrent severe cutaneous viral infections, autoimmunity and malignancy. Severe atopic dermatitis (AD) and DOCK8 deficiency share some clinical symptoms including eczema, food allergies, increased serum IgE levels and eosinophilia. Increased serum IgE levels are characteristic but not specific for allergic diseases. DOCK8 exhibits an unusual constellation of clinical features, and diagnosis can be confusing and difficult especially during early childhood.The clinical management of patients with moderate-severe AD and DOCK8 deficiency can be challenging and quite complex as they share many clinical features. Deficiency of DOCK8 protein is potentially, life threatening and only curable with bone marrow transplantation. Hence, the diagnosis of DOCK8 deficiency is critical and should be sought at an early stage to initiate definitive therapy.

A detailed review on the analytical methods in metabolomics used for biomarker discovery and workflow for data analysis is provided in **Chapter 1.** Metabolomics studies have identified several relevant biomarkers involved in complex clinical phenotypes using diverse biological systems. In this review, metabolomics strategies were compared and evaluated, to focus on the discovery of biomarkers that have diagnostic, prognostic, and therapeutic value, validated for monitoring disease progression and responses to various management regimens.

The work presented in this PhD thesis provides the development of a comprehensive metabolomics panel for biomarker discovery. Apart from cytokine biomarkers, metabolomics biomarkers differentiating DOCK8-deficient and AD patients were investigated. Furthermore, since these patients have food allergies in common, sensitization patterns were also evaluated.

In **Chapter 2**, a comprehensive cytokine profile revealed distinctive biomarkers that differentiate between the DOCK8-deficient and AD patients. The unique expression profile of various inflammatory cytokines in patients with DOCK8 deficiency vs AD likely reflects disease-specific perturbations in multiple cellular processes and pathways leading to predisposition to infections and allergies seen in these patients. Significant differential expression of the cytokines, CXCL10, CSF3, CCL22, CX3CL1, and TNF-A were identified in DOCK8 deficiency, which possibly contributes to increased susceptibility to infection and cancer. CXCL10 and TNF-A were up-regulated in DOCK8-deficient patients when compared to AD, possibly contributing towards increased susceptibility to infections and cancer. In contrast, epidermal growth factor (EGF) was significantly down regulated in a subgroup of DOCK8 deficient and AD when compared to the controls. The cytokine IL-31, is known to be elevated in patients with pruritus and IL-31 expression was found to be elevated in both our cohorts thus contributing toward pruritus common to both groups. The unique expression profile of various inflammatory cytokines in patients with DOCK8 deficiency vs. atopic dermatitis likely reflects upon the disease-specific perturbations in multiple cellular processes and pathways leading to a predisposition to infections and allergies seen in these patients. This data agrees with the role for EGF replacement therapy in EGF deficient individuals with AD as well as DOCK8 deficiency through a potential shared pathway.Hence, CXCL10, TNF-A and EGF were found to be signature cytokine biomarkers, leading to early effective diagnosis and distinction of DOCK8 deficiency from AD.

C**hapter 3** describes the development and validation of a comprehensive targeted metabolomics method for a panel of 225 clinically relevant metabolites on Liquid chromatography-tandem mass spectrometry (LC-MS/MS), a powerful analytical technique that combines the separating power of liquid chromatography with highly sensitive and selective mass spectrometry. The potential clinical usefulness of this method was evaluated on different biological matrices such as whole blood, serum, dried blood spots (DBS) and murine tissues. The sensitivity, selectivity, stability and linearity of this method was also

studied under defined preparation and analysis conditions. The method was tested and validated on eight different groups of patients with a known diagnosis of Inborn errors of metabolism (IEMs), which are rare genetic or inherited metabolic diseases resulting from an enzyme defect in biochemical and metabolic pathways, presenting with neurologic signs in newborns. The applicability of the method was also tested on various rat tissues to understand the metabolic tissue distribution pattern. The advantages of the developed method are small volume requirement, clinical relevance and cost effectiveness.

In **Chapter 4**, metabolomics based biomarkers were investigated using chemical isotope labeling liquid chromatography-mass spectrometry (CIL LC-MS) that is capable of distinguishing DOCK8-deficient from AD patients. The metabolomics profiles revealed seven positively identified metabolites through binary comparisons between DOCK8-deficient and AD patients. Aspartic and 3-hydroxyanthranillic acids were up-regulated in DOCK8 deficiency, whereas hypotaurine, guanosine and the dipeptides leucyl-phenylalanine and glycyl-phenylalanine were down-regulated. Hypotaurine, 3-hydroxyanthranillic acid and glycyl-phenyalanine were identified as potential biomarkers specific for DOCK8 deficiency.

In **Chapter 5**, In chapter 5 a comparative analysis of food and inhalant allergens, metabolomics and cytokines profiles was conducted in allergic patients with DOCK8-deficiency and AD. Serum samples from DOCK8-deficient and AD patients showed different and overlapping sensitization patterns; inparticular, the cat allergen (rFel d1) was significantly up-regulated in AD patients when compared to DOCK8-deficient patients. LC-MS, followed by multivariate analysis revealed distinct signature profiles of dysregulated metabolites in the DOCK8-deficient and AD patient groups. Both cohorts commonly showed altered amino acid metabolism and taurine & hypotaurine metabolism pathways. DOCK8-deficient patients demonstrated perturbed tryptophan metabolism pathways whereas in AD patient's caffeine metabolism pathways was altered. Food and inhalant sensitization in DOCK8 deficiency and atopic dermatitis induce distinct metabolomic and cytokine profiles suggesting both shared as well as unique mechanisms mediating these disorders

In summary, the newly developed metabolomics method shows a great potential to discover novel biomarkers for various diseases. Chemokines, CXCL10, TNF-A and EGF were identified to be signature cytokine biomarkers differentiating DOCK8 deficient and atopic dermatitis patients.DOCK8 deficiencies appear to have a distinctive metabolomics profile characterized by significant differential over expression of 3-hydroxyanthranillic acid,

aspartic acid and hyptotaurine. The work presented in this thesis provides an important contribution towards the development of biomarkers that have diagnostic, prognostic and therapeutic values for monitoring disease progression and responses to various management regimes. The complex nature of these diseases suggests that no single biomarker will be sufficient to meet the clinical needs of such patients. Future work involving the evaluation and validation of these biomarkers in larger cohorts involving other Omics will be essential before their clinical application is accepted.

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LIST OF ABBREVIATIONS

AD - Atopic dermatitis

ACOD1 - Aconitate Decarboxylase 1

AcN –Acetonitrile

ADA - Adenosine deaminase

AMP - Adenosine monophosphate

ATP - Adenosine triphosphate

ADSL - Adenylosuccinate lyase deficiency

ANOVA - Analysis of Variance

AUC - Area under the curve

RBC - Red blood cells

BCKDHA - Branched Chain Keto Acid Dehydrogenase E1 Subunit Alpha

BCKDHB - Branched Chain Keto Acid Dehydrogenase E1 Subunit Beta

CSF - Cerebrospinal fluid

COMET - Consortium for Metabonomic Toxicology

CE - Collision energy

C5DC - Glutarylcarnitine

CE-TOF - Capillary Electrophoresis Time Of Flight Mass Spectrometer

CSF3 - Colony stimulating factor 3

CXCL10 - C-X-C motif chemokine 10

CXCL3 - Chemokine (C-X-C motif) ligand 3

CIL LC-MS - Chemical isotope labeling liquid chromatography-mass spectrometry

DOCK8 - Dedicator of cytokinesis 8

DBS - Dried blood spots

DBT - Dihydrolipoamide branched chain transacylase

DM - Diabetes mellitus

DNA - Deoxyribonucleic acid

DNCB - Dinitrochlorobenzene

DnsCl - 12C-dansyl chloride

EPAS1 - Endothelial Per-Arnt-Sim (PAS) domain protein 1

EGF- Epidermal growth factor

EGFR - Estimated glomerular filtration rate

ELISA - Enzyme-linked immunosorbent assay

ETF - Electron transfer flavoprotein

ETFDH - Electron transfer flavoprotein dehydrogenase.

ESI - Electrospray ionization

FMLR - Fast maximum likelihood reconstruction

FWHM - Full width at half maximum

FEV1 - Forced expiratory volume in one second

FT-ICR - Fourier transform ion cyclotron resonance

FC - Fold change

FAH - Fumarylacetoacetate hydrolase

FBS - Fetal bovine serum

FDA - Food and drug administration

FDR - False discovery rate

FGF2 - Fibroblast growth factor 2

GMAPS™ - Global metabolomic assisted pathway screen

GC-MS - Gas chromatography-mass spectrometry

GCSF - Granulocyte colony stimulating factor

GA - Glutaric acidemia

GAGs - Glycosaminoglycans

GC - Gas chromatography

GCDH - Glutaryl-CoA dehydrogenase

HMG-CoA - 3-hydroxy-3-methylglutaryl coenzyme A

HbA1C - Hemoglobin A1c

HIES - Hyper-IgE syndromes

HSV - Herpes simplex virus

HPV - Human papillomavirus

HRP - Horse radish peroxidase

HPLC - High Performance Liquid Chromatography

HSCT - Hematopoietic Stem Cell Transplantation

HS-GAG - Heparan sulfate glycosaminoglycans

3-HAA - 3-Hydroxyanthranillic acid

IEM - Inborn error of metabolism

IPA - Ingenuity Pathway Analysis

IR - Insulin resistance

IL31 - Interleukin 31

ICH - International Conference on Harmonization

ISAC - ImmunoCAP Solid-Phase Allergen Chip

IQR - Interquartile range

IgE - Immunoglobulin E

IFNG - Interferon Gamma

IRB - Institutional Review Board

IS - Internal standard

ISU - ISAC standardized units

KFSHRC - King Faisal Specialist Hospital and Research Centre

LC - Liquid chromatography

LC-MS - Liquid chromatography-mass Spectrometry

LEAP - Learning Early About Peanut Allergy

LGPC - Linoleoyl-glycerophosphocholine

LCLs - Lymphoblastoid cell lines

LLOQ - Lower limit of quantification

MS - Mass-spectrometry

MAS - Magic Angle Spinning

mGFR - Measured glomerular filtration rate

MCV - Molluscum contagiosum virus

MDC - Macrophage-derived chemokine

MMA - Methylmalonic acidemia

MCM - Methylmalonyl CoA mutase

MCEE - Methylmalonyl-CoA epimerase

MSUD - Maple syrup urine disease

MRM - Multiple reaction monitoring

MeOH - Methanol

MET-IDEA - Metabolomics Ion based Data Extraction Algorithm

MADD - Multiple acyl-CoA dehydrogenase deficiency

MCEE - Methylmalonyl-CoA epimerase

MODY - Maturity-onset diabetes of the young

MSEA - Metabolite Set Enrichment Analysis

m/z - Mass to charge ratio

MIM- Mendelian Inheritance in Man

NMR - Nuclear magnetic resonance

NGS - Next generation sequencing

NANA - N-acetylneuraminic acid

NC - Normal control

NS- Not significant

OPLS - Orthogonal Partial Least Squares

OCT1 - Organic cation transporter 1

OGTT - Oral glucose tolerance test

PCA - Principal Component Analysis

PC - Principal component

PGM3 - Phosphoglucomutase 3

PBMCs - Peripheral blood mononuclear cells

PBS - Phosphate buffered saline

PA - Propionic acidemia

PKU - Phenylketonuria

PID - Primary immune deficiency

PDE4 - Phosphodiesterase 4

PLS - Partial Least Squares

pKa- Ionization Constant

PLS-DA - Partial least squares discriminant analysis

QC - Quality control

Q-TOF - Quadrupole time-of-flight

QqQ - Triple quadruple

REIMS - Rapid Evaporative Ionization Mass Spectrometry

ROC - Receiver operating characteristic

RIPA - Radio Immuno Precipitation Assay

RPM - Round per minute

RSD - Relative standard deviation

RT - Retention time/Room temperature

RBC- Red blood cells

SIR - Selected ion recording

SRM - Selected reaction monitoring

STAT3 - Signal transducer activator of transcription 3

SD - Sprague Dawley

SCORAD - Severity Scoring of Atopic Dermatitis

SDS-PAGE - Sodium dodecyl sulfate- Polyacrylamide gel electrophoresis

SEM - Standard error of mean

SAT1- Spermidine/ spermine N (1) acetyltransferase 1

SHGP - Saudi Human Genome Project

SVM - Support vector machine

SMS- Spermine synthase

T2DM - Type 2 diabetes mellitus

Th - T-helper cells

TNFA - Tumor necrosis factor alpha

TIC - Total ion chromatograms

TALDO1 - Transaldolase 1

TBM - Tuberculin meningitis

TKT - Transketolase

TSLP - Thymic stromal lymphopoietin

TAT- Trans-Activator of Transcription

TUG1 - Taurine-upregulated gene 1

UPLC - Ultra performance liquid chromatography

USFDA - United states food and drug administration

VOC - Volatile organic compounds

VZV - Varicella-zoster virus

VLCAD - Very-long-chain acyl-coenzyme A dehydrogenase deficiency

VAS - Visual Analogue Scale

WES - Whole exome sequencing

WBC - White blood cells

XCMS – Gas/ liquid chromatography mass spectrometry

CHAPTER 1: INTRODUCTION TO METABOLOMICS

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1.1. Introduction

Hyper immunoglobulin E (IgE) syndrome [HIES] are a group of primary immunodeficiency disorders with highly elevated IgE levels, recurrent staphylococcal skin abscesses, eczema and pulmonary infections. In 1966, Davis et al, described two girls suffering from staphylococcal abscesses, eczema and chronic pulmonary disease, naming this entity as 'Job's Syndrome'. HIES is inherited in both autosomal dominant (AD) and recessive (AR) patterns. Most of the AD HIES phenotypes are found to be linked to mutations in the (signal transducer and activator of Transcription 3*) STAT3*, whereas autosomal recessive hyper-IgE syndrome (AR-HIES) is a serious inborn error of primary immunity caused by mutations in *DOCK8* and less frequently *PGM3.* Mutations on the Dedicator of Cytokinesis 8 (DOCK8) were found to account for the patients with AR-HIES. Homozygous and heterozygous mutations are reported with frequent large deletions. These patients have an absent or loss of function of the DOCK8 protein.(1) (2) DOCK family consists of 11 members and are classified in four subfamilies (DOCK-A,B,C and D) based on their sequence homology and substrate specificity.(3) DOCK8 is a member of the DOCK180 superfamily of guanine nucleotide exchange factor, which interacts with Rho GTPases, DOCK8 has a typical guanine nucleotide exchange factor (GEF) for small G proteins of Rho family such as Rac and Cdc42 DOCK8 proteins regulate actin cytoskeleton, cell adhesion and migration. (4) DOCK8 mainly has two related conserved protein domains DHR1 and DHR2, wherein the DHR1 domain binds to phosphatidylinositol-3,4,5-triphosphate (PtdIns 3,4,5)P3 and mediates the recruitment of DOCK proteins to the plasma membrane, whereas the DHR2 domain functions as a GEF for CDC42.(5)

Atopic dermatitis is one of the common pediatric chronic inflammatory skin disease and certain food-allergens and nutrients are closely related to the development and severity of the disease. It is a common chronic or relapsing eczematous dermatitis characterized by intense

pruritus and occurs primarily in infants and children. Atopic dermatitis over laps with HIES in terms of eczema, eosinophila, food allergies and characteristically increased serum IgE levels.

Combining high throughput experimental "OMIC based" techniques such as genomics, proteomics, and metabolomics with computational techniques such as bioinformatics and computer simulations enable us to understand specific targets in biological systems. Metabolomics relates to small molecule identification and quantification, and the resulting network interactions, which represent the individual's functional genome. The entire qualitative collection of metabolites in a biological sample is called the "metabolome" which is very dynamic: small molecules are continuously absorbed, synthesised, degraded and interact with other molecules, both within and between biological systems, and with the environment. Significant environmental and clinical disturbances can be monitored at the metabolic level, in an array of different pathways that are crucial for cellular homeostasis.(6) The study of metabolites started more than 100 years back; metabolites were first measured in the pioneering work of the early biochemist, Sir Hans Krebs, who discovered the citric acid cycle and the urea cycle. The term metabolomics was first used in 1998 by Steve Oliver and colleagues.(7) Metabolomics is the comprehensive and simultaneous profiling of metabolites, which evaluates their effective changes resulting from different conditions such as diet, lifestyle, genetic, or environmental factors. Metabonomics as a subset of metabolomics is defined as "the quantitative measurement of the dynamic 'especially inter-population' multi parametric metabolic responses of the living systems to pathophysiological stimuli or genetic modification with a particular focus on environmental (including nutritional) stress.(8)

Developments in instrumentation technologies such as Nuclear magnetic resonance (NMR), Mass spectrometers, (MS) and Ultra performance liquid chromatography (UPLC) in combination with bioinformatics, have paved the way to an improved understanding of the

metabolome.(9, 10) Over the past decade the application of metabolomics platforms in clinical research, especially MS based metabolomics, has increased as illustrated in **Error! R eference source not found..**

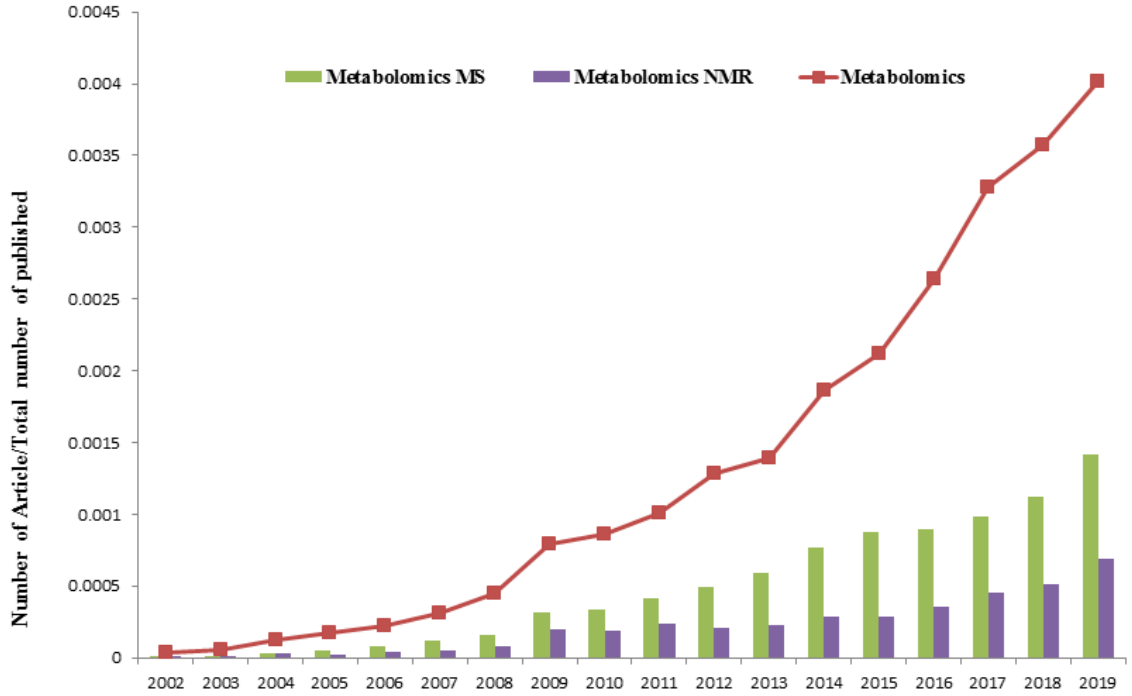


Figure 0.1: Bibliographic searches in PubMed containing the keyword metabolomics (as of November 2019). A total of 15230 articles were identified, where the dataset was mined using the search parameter mass spectrometry or NMR, and normalized to total number of published articles in general.

Since metabolites represent the end products of cellular biochemical and physiological processes, studying the metabolome in combination with transcriptomics and proteomics will make more sense to understand disease pathogenesis and mechanism of intervention. Many human diseases are heterogeneous in their clinical presentation and etiology and have a complex molecular pathogenesis. Thus, genetic and high-throughput molecular profiling can be a decisive factor in developing a more comprehensive and informative classification of diseases.(11) In systems biology, patient phenotypes and pathological status have opened doors to the application of metabolomics in drug discovery processes and personalized medicine.(12) For example, Lindon et.al (2005) reported The Consortium on Metabonomic Toxicology (COMET) project, launched to assess the use of NMR-based metabolomics for determining the early toxicity of drug candidates at preclinical developmental stages and for establishing systems predicting target organ toxicity. (13). The panel of metabolites included in the metabolome represents a distinct metabolic fingerprint or signature, indicative of its current state. Such a profile might also include any molecule that humans ingest, catabolize/metabolize, or come in contact with such as peptides, lipids, amino acids, carbohydrates, organic acids, nucleic acids, hormones, vitamins, minerals, and many other molecules with molecular weight <1500 Da. (14) The epigenome and the metabolome can be quite diverse regarding chemical, biological, environmental and physiological complexity.

Personalized health is an ancient approach that existed in Chinese medicine and Ayurveda, in which the mode of treatment involved a personalized approach to the patient. Hence diagnosis and treatment can be improved by blending biology with medicine through the integration of Western and Eastern knowledge.(15) In the United States, among the top 10 highest selling drugs, only up to 25% of the people who use these drugs get the benefit. (imprecision medicine). Statins (3-hydroxy-3-methyl- glutaryl coenzyme A (HMG-CoA) reductase inhibitors) are used to lower cholesterol, in patients treated for high cholesterol, but

only a very low margin of 2% of those patients get the benefit of the drug. Some classical drugs that had been developed through clinical trials involving participants from a western population can be even harmful to certain ethnic groups.(16) Several leading countries in the developed world, such as the USA, have established a precision medicine initiative.
The NIH funded “All of US Research Program;(https://allofus.nih.gov)” is a historic effort that aims to gather clinical and molecular data from one million or more Americans to accelerate research and improve health, taking into account individual differences in lifestyle, environment, and biology. In Saudi Arabia, as an initiative towards precision medicine, the government is taking advantage of the next generation sequencing (NGS) technology and has initiated the Saudi Human Genome Project (SHGP) (http://shgp. kacst.edu.sa) that targets sequencing the whole genome of 100,000 Saudis within 5 years. Unlike classical clinical trials, precision medicine requires several ways of monitoring interventions, which encourages researchers to characterize the genetic and environmental factors that impact on a person's reaction to a particular intervention. These individual factors need a high throughput, comprehensive, and quantitative platform to personalize the diagnosis, which will subsequently reflect on the personalized utilized interventions.

Finding metabolomic signature profiles may be useful in clinical care and biomarker discovery for diseases like combined immunodeficiencies and primary immunodeficiencies. Currently, a few targeted metabolic profiles such as acylcarnitines, amino acids, very long chain fatty acids, and organic acids are routinely performed in newborn screening and biochemical genetics laboratories worldwide.(17-20) Few laboratories in the world have adopted a comprehensive clinical metabolomics based approach in the evaluation of patients suspected of having a metabolic or multifactorial disease. Nutritionists and dietitians are re exploring metabolomics to identify novel ways to treat and prevent nutrition related diseases such as obesity, diabetes, and some inborn errors of metabolism. (IEM).(21, 22) Therefore,

there is a strong need for a comprehensive metabolomic database that encompasses many physiological health and disease states identified in a large cohort of well-phenotyped individuals.(23, 24)

A combined untargeted metabolomics analysis linked with DNA variants identified by "Whole Exome Sequencing" (WES) in healthy volunteers, revealed a range of complementary molecular and metabolic abnormalities.(25) In particular, metabolomic analysis helped clarify the clinical significance of DNA variants thought to be pathogenic. It also identified metabolic abnormality related DNA variants that were not flagged on WES. These combined approaches including personalized medicine have contributed toward disease risk analysis, disease monitoring, and drug management, which have the potential to transform clinical practice and treatment efficacy.(26) In humans, the largest list of metabolites available is the Human Metabolome Database, developed by David Wishart and his group in Canada defining more than 40,000 metabolites.(27)

In this review, we provide an overview of the metabolomics based workflow of personalized medicine studies: sample preparations, pros, and cons of different methodologies and separation techniques involved and discuss its contribution toward the field of precision medicine and biomarker discovery. Precision metabolomics may provide a snapshot of the current state of health and actionable information to advance clinical decision-making.

1.2. Clinical sample collection and preparation methods

High quality results are achieved in metabolomics when a reliable bio specimen is collected, processed appropriately, and a typical instrumental analysis method is set up to identify the metabolic biomarkers in a cost effective manner. Metabolism operates in constant flux, where metabolite concentrations change rapidly compared to other cellular biomolecules, hence following standardized procedures for sample collection and storage are vital for sample quality and the success of metabolomics experiments. The choice of the sample is dependent

on many factors including the biological question posed and the availability of an appropriate specimen. Several metabolites may be affected by different factors such as medication, environment, fed versus fasting state, time and way of sample collection, storage and the processing time. For example, repeated freeze/thawing of cerebrospinal fluid (CSF) has been reported to result in changes in transthyretin peptide levels.(28) Failure to adhere to these standardized procedures could lead to false positive or false negative results. The less invasive sample types, such as urine and blood, are the most ideal and available samples for routine clinical analyses. A significant advantage of metabolomics is the capability of measuring a broad spectrum of metabolites in urine, serum, plasma, feces, sweat, saliva, blood, ascetic fluid, bronchial washes, prostatic secretions, soft tissues, dried blood spots (DBS), and CSF. In some applications, exhaled breath condensates are being used as a bio specimen for metabolic studies in asthma and airway inflammation,(29) as shown in **Error! R eference source not found.**. The sample extraction method should be capable of targeting the maximum amount and classes of metabolites present in the target sample by pre-concentrating the extracts and removing the interfering compounds. Sample preparation depends on the range of instrumentation used in metabolomics, where LC-MS/MS and NMR are the most commonly used techniques, requiring a clean sample with a compatible solvent before the analysis. Sample preparation for MS is accomplished in two stages; quenching metabolism and extracting metabolites.

Quenching is the process of arresting or stopping the cellular metabolic activity with limited cell membrane damage. If quenching is not performed, the sample analyzed will not be representative of the metabolome at the point of sampling. Snap freezing in liquid nitrogen followed by sample storage at −80°C is an ideal and frequently used quenching methods. Other techniques like freeze-clamping, acid treatment, or using salt containing aqueous methanol at low temperatures have also been used for metabolism quenching.(30) Extraction

is the process of isolating the metabolites from the sample using organic solvents like methanol, chloroform, and acetonitrile depending on the matrix (serum, plasma, urine). The extraction of metabolites from tissues can be achieved by grinding them manually or by using an electric tissue homogenizer. Solvent extraction is the most commonly used mode of extraction for tissues due to higher extraction efficiency and minimum ionization suppression in LC-MS analysis.(31, 32) The success of an analytical procedure depends on the quality of sample preparation, extraction procedure, cell type, chemical properties, and the reactivity of the cellular enzymes. The targeted groups of polar and/or non-polar metabolites are extracted by choosing solvents that are watersoluble or lipid soluble. NMR-based analysis requires a much simpler sample preparation without chemical derivatization. Thus, it is specifically useful for metabolic profiling of intact bio fluids and semisolids like intact cells or tissues. Hence, experimental procedures should be standardized and optimized to quench, extract and store the sample in an identical manner, because small differences in sample processing can have a significant effect on the sample quality and validity of the data acquired.

1.3. Analytical methods in metabolomics

A striking advantage of metabolomics is the easy transition from a profiling technology to specific clinical assays accessible for small molecules. The ultimate goal in metabolomics is to generate a reliable profile, for the maximum number of metabolites in a biological sample. However, the targeted metabolites have different chemo physical properties, such as hydrophobicity, acidity, charge, pKa, and size. Hence, simultaneous separation of all the metabolites in a single assay is quite challenging. An extensive metabolomics analysis can yield beneficial structural and sensitive quantitative data for complex biological samples. NMR and LC-MS/MS are the most commonly used spectroscopic techniques in metabolomics. LC- MS/MS is the universal technique, because of its combinability with

other chromatographic techniques that allow the separation of the vast majority of molecules with very few limitations

Table 0.1: Application of metabolomics in various clinical disorders.

Disease /Condition	Metabolomics platform		Biomarkers/Major findings	Matrix	Platform	Refer-ence
	Targeted	Untargeted				
Periodontal disease		√	Inosine, putrescence, xanthine	Gingival Crevicular fluid	LC-MS/MS	(33)
			L-Lactic acid	CSF	LC-MS/MS	(34)
Tuberculous meningitis (TBM)	√		Serotonin, kynurenine	Serum	LC-MS/MS	(35)
Dengue fever		√	Altered lipid metabolism, apoptosis, and hyper phosphorylation	CSF	LC-MS/MS	(36)
Alzheimer disease	√		Azelaic acid, glyceric acid, pseudouridine, 5-hydroxyhexanoic acid, tyrosine, phenylalanine	Urine	GC-MS	(37)
Post stroke depression	√		Uric acid, hypoxanthine, guanine, xanthosine, inosine, methylguanine	Urine	LC-MS/MS	(38)
Xanthinuria	√		δ –Valerolactam	Saliva	LC-MS/MS	(39)
Physiological stress		√	Phosphatidylethanolamines and phosphatidylcholines	Blood	GC-MS	(40)
Schizophrenia	√		Lysophoshatidylethanolamines and lysophosphatidylcholines	Plasma	LC-QToF-MS	(41)
Bipolar depression treated with Ketamine		√	Hypoxanthine, N4Acetylcysteine, N-acetylmethionine, Mannose, cortisol	Plasma	LC-MS/MS	(42)
Cystic fibrosis	√		2-phenylacetamide, phenyl pyruvic acid, tyrosine, adrenocorticotropic hormone, sphinganine, citric, and aconitic acids	Seminal plasma	LC-MS/MS	(43)
Infertile males with Kidney-Yang deficiency	√		lactate, glycoprotein, glucose, valine, proline, and pyruvic acid	Blood	NMR	(44)
Chronic heart failure	√		1,5-Anhydroglucitol	Saliva	LC-MSMS	(45)
Diabetesmellitus	√	√	Glycine, serine and threonine metabolism, steroid hormone biosynthesis, tyrosine metabolism,glycerophospholipid metabolism and fatty acid metabolism	Serum	LC-QToF-MS	(46)

Disease /Condition	Metabolomics platform		Biomarkers/Major findings	Matrix	Platform	Refer-ence
	Targeted	Untargeted				
Gestational diabetes mellitus			3-Indoxy sulfate, glycerophospholipids, free fatty acids and bile acids	Blood	LC-MS and NMR	(47)
Diabetesmellitus			Taurine,piperidine	Saliva	CE-ToF-MS	(48)
Oral cancer	√		Suberic acid, docosadienoic acid, tri hexose and tetra hexose	Sweat	LC-QTOF	(49)
Lung cancer (screening)		√	Phospholipids	Plasma	LC-TOF-MS	(50)
Esophageal Squamous-cell carcinoma			Maltose, palmitic acid, glycerol, ethanolamine, glutamic acid, lactic acid, lysine and histidine	Blood	GC-TOF-MS	(51)
Lung cancer			Pipecolic acid,Asparagine, Proline, C14:1,Phe/try and Gly/Ala ratios	DBS	LC-MS/MS	(52)
Breast cancer (rapid detection)	√		Phosphatidylcholines	Plasma	LC-MS/MS	(53)
Breast, prostate and colorectal cancers			Cytidine-5-monophosphate,pentadecanoic acid	Tissue	GC-TOF-MS	(54)
Breast cancer			Galactinol,melibose, and melatonin	Tissue	GC-TOF-MS	(54)
Papillary thyroid carcinoma		√	Alanine,glutamate up regulated	Cell lines	LC-MS/MS	(55)
Apoptosis in cancer cells			2-Hydroxyglutarate	Blood	LC-MS/MS	(56)
Acute Myeloid leukemia	√		Glucose	CSF	NMR	(57)
Amyotrophic lateral sclerosis	√		Citric, Isocitric, lactic,2-aminopimelic acid	CSF	GC-MS	(58)
Glioma	√		Creatinine,creatine,glucose and ά-hydroxybutyrate	CSF/Plasma	LC-MS/MS / GC-MS	(59)
Amyotrophic lateral sclerosis	√		Leucine,isoleucine and keto-leucine	CSF/Plasma	LC-MS/MS / GC-MS	(59)
Parkinsondisease	√		Lysine metabolism in plasma and Kreb cycle in CSF	Plasma and CSF	TOF-MS	(60)
Mild Cognitive Impairment		√	Cholesterol and sphingolipids transport	Plasma and CSF	TOF-MS	(60)

Disease /Condition	Metabolomics platform		Biomarkers/Major findings	Matrix	Platform	Refer-ence
	Targeted	Untargeted				
Alzheimerdisease		√	CSF lactate, serum Glucose and valine in serum, urinary Taurine	CSF/serum/urine	NMR	(61)
Multiple sclerosis		√	N8-acetylspermidine	Plasma	LC-MS/MS	(62)
Synder-Robinson syndrome		√	Glycine, citrulline, creatinine, asymmetric dimethylarginine, symmetricdimethyl arginine and dimethyl glycine	Plasma	LC-QTOF-MS	(63)
Pediatric chronic kidney disease	√		Tyrosine and phenylalanine	Urine	NMR/GC-MS/Amino acid analyzer	(64)
Phenylketonuria	√		Lipid and carnitine levels	Plasma	LC-MS/MS	(65)
Healthy pregnant women		√	Low short chain fatty acids, Perturbed ethylacetate, octylacetate, glutamine	Feces/urine	GC-MS/ NMR	(66, 67)
Coeliac disease	√		Taurine, methanol, fumarate and tryptophan	Fecal extracts	NMR	(68)
Ankylosing spondylitis and rheumatoid arthritis	√		Butyrate, propionate, and acetate	Feces	NMR	(69)
Effect of exclusive enteral nutrition in Juvenile idiopathic arthritis	√		Decrease in butyrate fermentation and enhanced excretion of non-esterified fatty acids and bile acids	Feces	LC-MS/MS	(70)
Effect Of surgical and dietary weight loss therapyonobesity	√		Increased levels of lactate, long chain fatty acids, triglycerides, and low-density lipoprotein. Glucose, phosphatidylcholine, and high-densitylipo protein concentrations were low. Decrease in BCAA/AAA ratio	Blood	GC-ToF-MS and NMR	(70)
Polycystic ovary syndrome		√	Upregulation of alanine, choline, creatinine and phosphorylcholine	Tissue	NMR	(71)
Effect of Temozolomide Resistance	√		Reduced levels of succinic acid and phosphatidylcholine at birth, reduced levels of	Serum	GCxGC ToF	(71)

Disease /Condition	Metabolomics platform		Biomarkers/Major findings	Matrix	Platform	Refer-ence
	Targeted	Untargeted				
in Glioblastoma multiforme			triglycerides and antioxidant ether phospholipids, diminished keto leucine and elevated glutamic levels			
Dysregulation of lipid and amino acids in children with pre-diabetes type-1		√	Exhaled Nitric oxide and FEV1(Forced Expiratory Volume in the first second)	Breath condensate	NMR	(29)
Childhood Asthma	√		Propionyl-carnitine and Gama butyro- betaine	Plasma	ToF-MS	(72)
Propionic and Methylmalonic acidemia		√				

(Abbreviations: GC-MS: Gas Chromatography-Mass Spectrometry, LC-MS/MS: Liquid Chromatography-tandem Mass Spectrometry, LC-QToF-MS: Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry, CE-QToF-MS: Capillary Electrophoresis Quadrupole Time-of-Flight Mass Spectrometry, NMR: Nuclear Magnetic Resonance)

1.3.1. Nuclear magnetic resonance (NMR)

NMR characterizes molecules based on the principle that certain molecular atoms can absorb and resonate radiation at specific frequencies when placed in a magnetic field. NMR is nondestructive and independent of any analytical separation techniques because samples are relatively fast and easy to run at a low running cost. It is also possible to improve on the absolute quantitation by spiking samples with a known concentration of the standard internal compound. However, in NMR, the sensitivity and difficulty of annotating the metabolites signals to useful biological information is a major limitation, in addition to the lack of connectivity to separation techniques such as High performance liquid chromatography (HPLC), which separates and quantifies compounds using operational pressures, or. Gas chromatography (GC), which separates and analyzing compounds that can be vaporized without decomposition or Capillary electrophoresis (CE) which employs electrolyte solution under the influence of an electric field, for separation.(73, 74) The slow convergence for computing the likelihood estimators was made faster through the introduction of an algorithm called Fast Maximum Likelihood Reconstruction (FMLR), where computation takes less than one minute for a single data set with far higher accuracy than previously possible.(75) A recent development, Magic Angle Spinning (MAS) NMR, has also been utilized for detection, and spatial mapping of metabolites in intact tissues and cells. This technique is often used to perform experiments in solid-state NMR spectroscopy and, more recently, liquid Proton nuclear magnetic resonance. The samples are spinning at the magic angle with respect to the direction of the magnetic field; the normally broad lines become narrower, which increases the resolution for better identification and analysis of the spectrum. Andronesi et al presented a brain tumor biopsy based study, where they detected 16 metabolites within a short time of 45 min through molecular tissue characterization and fingerprinting.(76) Since, Breathomics is the metabolomics study of breath condesates or

exhaled air, an NMR-based study, done by coupling metabolomics and breathomics revealed a marked difference between healthy and asthmatic children.(29)This technology has also been used successfully for the determination of biomarkers for clinical conditions such as phenylketonuria and multiple sclerosis. **Error! Reference source not found.** (51, 55)

1.3.2. Gas chromatography-mass spectrometry (GC-MS)

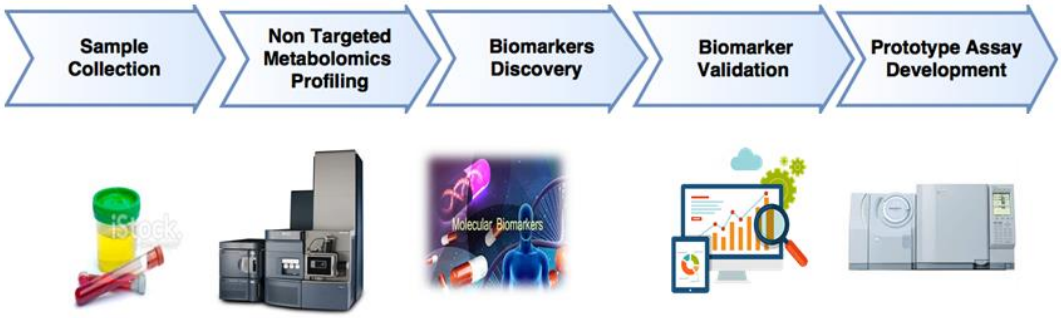
GC-MS is one of the most frequently used techniques in metabolomics for comprehensive analysis of primarily volatile and low molecular weight metabolites because of its low running cost, performance stability, result reproducibility and high peak capacity. Combining mass spectrometry with a separation technique such as GC amplifies the instrument's specificity, sensitivity, and reproducibility. GC-MS is a fundamental technique because compound identification is greatly facilitated by extensive, easily searchable databases of molecular fragmentation. The analysis of volatile compounds that are not easily detectable by LC-MS/MS is possible with high separation efficiency and reproducible retention times. GC-MS can be used only for volatile and thermally stable compounds, most of them requiring chemical derivatization, which prolongs the processing and analysis time. The similarity of molecular fragmentation patterns for structural isomers may make compound identification difficult in GC-MS.(77-79) Time-of-Flight (TOF) is a mass Analyser, wherein the ions are extracted (or produced) in short bursts or packets within the ion source and subjected to an accelerating voltage. These ions then 'drift' or 'fly' down an evacuated tube of specific length.The flight times are correlated against at least two known masses from an infused tune compound allowing a simple conversion to obtain a typical abundance versus mass to charge (m/z) spectrum. GC/MS system coupled with TOF analyzer provides rapid accumulation of spectra leading to excellent reproducibility and better signal-to-noise characteristics in a small volume of sample. For example, Miyamoto et.al (50) used GC- TOF-MS to examine the prognosis for lung cancer through biomarker discovery, as shown in Error! Reference s

ource not found.**Error! Reference source not found.**(45) The GC-MS method has undergone a few modifications over the past decade, with respect to the mass analyzers used such as TOF and the triple quadruple (QqQ). The latest advancement is two-dimensional chromatographic separation system (GCxGC-MS), which has been used recently for biomarker selection of candidates from untargeted metabolomics analyses.(80).TOF analyzers are preferred because of their higher scan speed, sensitivity, compatibility with GC and high resolution as a new analyzer, resulting in better characterization of unknown metabolites by using accurate masses. This technique has also proved to be useful in studying inborn errors of metabolism and identifying early biomarkers for a variety of other human disorders such as polycystic ovary syndrome, gestational diabetes mellitus, pediatric chronic kidney diseases, mild cognitive impairment, Alzheimer, and breast, thyroid and lungs cancer as shown in Error! Reference source not found.. (52, 53)

1.3.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

LC-MS/MS is not only a powerful analytical technique that combines the separating power of liquid chromatography with the highly sensitive and mass spectrometry, but also an important analytical tool in metabolomics, where these two universal techniques accommodate the chemical diversity of metabolites. (Hydrophobicity, charge, pKa, size, stability). Analysis of a wide range of metabolites, ranging from high to low molecular weight and from hydrophilic to hydrophobic can be performed by selecting the appropriate stationary phase and mobile phase, such as using ion-pairing reagent with phosphor sugars, ATP, AMP.(81, 82) Electrospray ionization (ESI) is the source of choice for metabolomics in LC-MS/MS because it can ionize a wider spectrum of molecules softly. However, ESI lacks quantitative capabilities due to the matrix or ion suppression effects.LC-MS/MS based metabolomics analysis of polar and high molecular weight metabolites does not require derivatization. Ultra pressure liquid chromatography (UPLC) is a separation technique applicable for particle less

than 2µm in diameter to acquire better resolution, speed, and sensitivity compared with high-performance liquid chromatography (HPLC). The introduction of UPLC (Waters Corporation) in 2004, added advantages like shorter run time, improvement in sensitivity, and high peak capacity to LC-MS based metabolomics. LC-MS/MS is now widely used in clinical metabolomics for precision medicine and disease biomarker discovery applications such as cancer and diabetes mellitus as shown in **Table 1.1.** (47, 53-55) These discovered biomarkers will not only contribute to the development of precision diagnosis and treatment but also to personalized susceptibility disease assessment, health monitoring, and preventative medicine. Fourier transform ion cyclotron resonance (FT-ICR) remains as the MS method with the greatest mass resolving power of 1,000,000 FWHM (Full Width at Half Maximum), using m/z measurement based on cyclotron frequencies of the ions in a fixed magnetic field. The Orbitrap MS combines premium dual- pressure linear ion trap with a novel high field Orbitrap™ mass analyzer to create an analytical instrument, which is easier to use and maintain and can easily be coupled to LC by ESI source. Orbitrap based metabolomics is regularly employed in clinical metabolomics analysis which had been applied successfully in the diagnosis of various inborn errors of metabolism such as aromatic L-amino acid decarboxylase deficiency, adenylosuccinate lyase deficiency (ADSL) deficiency, secondary ADA (adenosine deaminase) deficiency in sickle cell disease as well as the study of benznidazole efficacy against trypanosomacruzi.(83-85)



Cancer	Plasma	UPLC/TOF/MS	Up regulated Phospholipids and bile conjugates	Specifically classified phenotype samples	LC-MS
Methylmalonic and Propionic Acid	Plasma	UPLC-MS and MS/MS	Proionylacarnitine Gama butyrobetaine	Specifically classified phenotype samples	LC-MS
Childhood Asthma	Breath	NMR platform was used for profiling	Nitric acid and FEV1 (forced expiratory volume in one second)	Specifically classified phenotype samples	NMR
Acute kidney Injury	Urine	UPLC-MS and MS/MS	Homovanillic acid sulphate	Specifically classified phenotype samples	LC-MS

Figure 0.2: Examples of biomarker development pipelines (Abbreviation: LC-MS/MS-Liquid chromatography tandem mass spectrometry, NMR-Nuclear Magenetic Resonance.)

1.4. Metabolomics biomarker discovery pipeline

The biomarker discovery pipeline uses a combination of technologies to capture the data, which is then translated to select for biomarkers that most reliably detect the disease as shown in **Error! Reference source not found..** The pipeline begins with the analysis of a b iospecimen on a targeted analysis platform using a method, which is robust and reproducible to ensure consistent performance. The biomarker candidates that emerge are further evaluated or validated across a large number of samples in the next stage of the pipeline. Validation must be sufficiently robust to achieve a high level of performance for routine clinical use and ensure that the data is of the highest quality regarding the clinical question posed. Biomarker candidates at this stage can be measured accurately with sensitive and reliable methods, which have been proven to distinguish patients from healthy subjects. Since mGFR (measured glomerular filtration rate) is an impractical procedure in routine clinical practice, serum creatinine and eGFR (estimated glomerular filtration rate) were the only traditional metabolic biomarkers of renal function regardless of the specific underlying kidney disease. Hence, despite their distinct molecular causes, acute, and chronic renal injuries were recognized by the same biomarkers which do not inform the pathophysiology of these disorders.

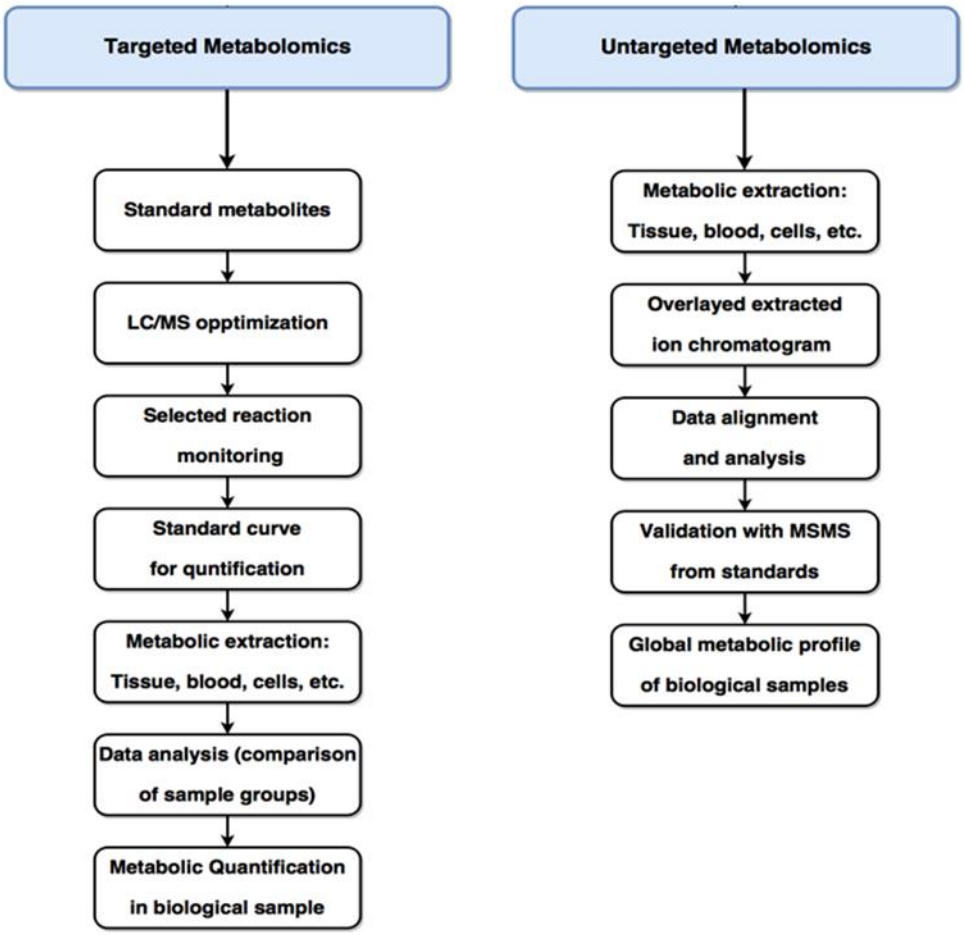


Figure 0.3: The targeted and untargeted workflow for LC-MS based metabolomics

Alternatively, a metabolome wide association study done in kidney function and disease identified six relevant analytes “C- mannosyltryptophan, pseudouridine, N-acetylalanine, erythronate, myoinositol, and N-acetylcarnosine,” which together have been referred to as “accuGFR”TM. (86) The discovery of anti-phospholipase A2 receptor antibodies which are also used as a prognostic biomarker of membranous nephropathy already impacted on the management of patients with this kidney disease.(87) Biomarkers evaluation in different matrices in patients with complex disorders that lack specific diagnostic markers may allow accurate and more confident diagnosis. For example, in Alzheimer disease, cholesterol, and sphingolipids transport are perturbed in both CSF and plasma, in contrast to perturbed lysine and Krebs cycle metabolism found in individuals with mild cognitive impairment.(88)

A metabolomics study can be performed either in a targeted or untargeted manner depending on the MS platform, and the data processing strategy as summarized in **Error! Reference s ource not found.** . Untargeted analysis covers and attempts to identify all detected peaks above the noise threshold using scan mode and utilizes both annotated and unannotated peak information for statistical analysis. The comprehensive analysis of the detected metabolites in a biological sample using this approach is used mainly for “clinically relevant” biomarker discovery. On the other hand, the targeted analysis seeks only known metabolites detected by selected reaction monitoring (SRM) using triple quadruple tandem mass spectrometry or selected ion recording (SIR) by GC-MS to validate a biological hypothesis regarding levels of known and expected endogenous metabolites.

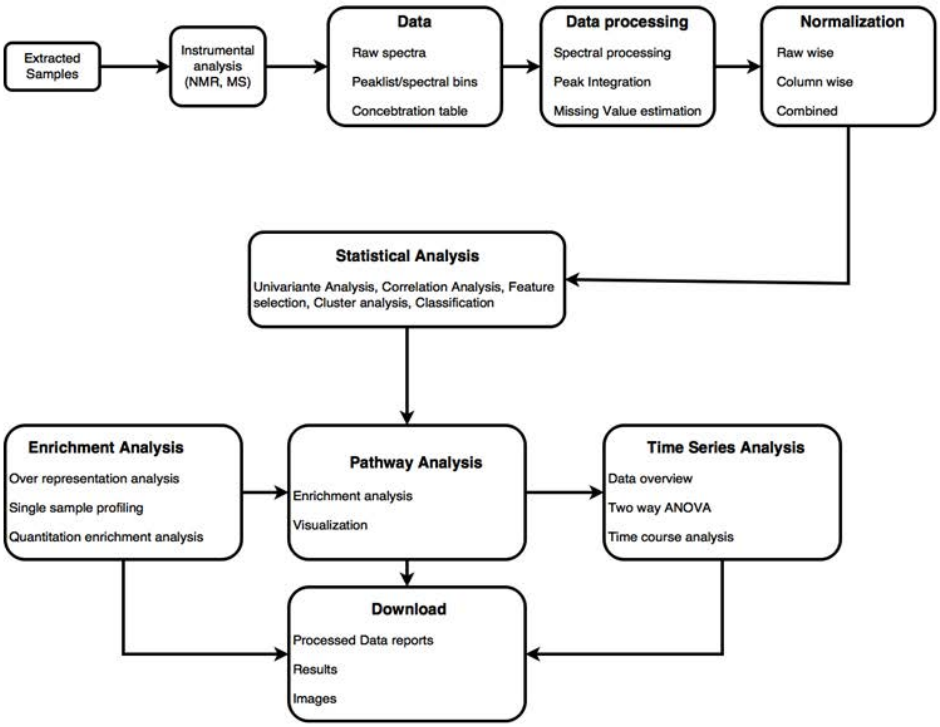


Figure 0.4: The targeted and untargeted workflow for LC-MS based metabolomics

Since the targeted strategy is based on the prior biochemical knowledge of the endogenous metabolites, and the sample content, biomarker discovery is achieved by mapping the absolute metabolic changes to the pathophysiological knowledge or integrated with other "omics" datasets.(22, 89, 90) Performing an untargeted metabolomics study requires an advanced analytical method, automated spectral data processing, and biological data interpretation and hypothesis generation.(90) High resolution tandem mass spectrometers such as QTOF, FTICR, and Orbitrap are preferred instruments for untargeted metabolomics studies due to their ability to elucidate structural information and speed of data acquisition, whereas QqQ and conventional ion trap mass spectrometers have been used for targeted metabolomics studies.(92)

In an illustrative study of Chinese men with infertility and renal insufficiency, a metabolomic signature of 41 differentially expressed, potential biomarkers related to aromatic amino acids, tri carboxylic acid cycle, and sphingolipid biosynthesis and metabolism were identified. Therefore this panel of metabolites helped in the understanding of the pathogenesis of kidney disease associated with male infertility.(43) Also, multivariate analysis of an untargeted metabolomics study of urine samples from 41 atopic asthmatic children showed reduced excretion of urocanic acid, methyl imidazole acetic acid and an unknown metabolite resembling the structure of an Ile-Pro fragment.(93)

1.4.1. Chemometric untargeted profiling

The metabolomics data obtained from patient samples and the relevant disease pathophysiology are highly complex and require computational approaches to convert the raw data into biological knowledge and connect them in a meaningful way. Data mining of this vast array of metabolites is a major challenge, and various statistical techniques are required to manage the data and follow a defined workflow to avoid any misinterpretations

Error! Reference source not found.. (94)Processed data obtained from metabolomics run has to b e uploaded to a web based or in-house built software program where data processing occurs in which various forms of raw data are converted into data matrices suitable for statistical analysis. Data normalization corrects any unwanted systematic variations and adjusts the data by identifying outliers and missing values. The choice of pretreatment method depends on the biological question that needs to be answered. The goal of data analysis is to determine relevant features and detect unique patterns, to assess differences between the phenotypes and facilitate classification/prediction. Multivariate or univariate analyses are usually performed for statistical analysis, where the multivariate analysis simultaneously analyzes all the variables, whereas univariate analysis is best suited for two groups of variables. Principal Component Analysis (PCA) is the most frequently used approach for data mining as an unsupervised approach and forms the basis of multivariate analysis.(95-97)

Pathway analysis helps to enhance metabolite set enrichment analysis for pathways by considering the structures of pathways and dynamic pathway visualization. Pathway analysis along with bioinformatics study helps greatly in biological interpretation and validation of computationally derived results.(98) The purpose of enrichment analysis is to test the validity of the biological groups (pathways, genotype) of metabolites that are significantly enriched in the queried data. Ingenuity Pathway Analysis (IPA) (QIAGEN bioinformatics, Redwood city, CA), is a software application that enables analysis, integration, and understanding of the complex “Omics” datasets, which in turn provides vital information on the candidate biomarkers in the context of metabolomics and proteomics experiments

1.4.2. Targeted metabolomics profiling

In the chemometric approach for data interpretation, PCA is unsupervised and multivariate analysis is based on projection methods. Each principal component (PC) is a linear combination of original data parameters, and the successive PC explains the maximum

amount of variance possible, not accounted for by the previous PCs. The principle of Partial Least Squares (PLS) is very similar to PCA, except for a labeled set of class identities being added. The PLS algorithm maximizes the covariance between the X variables and the Y variables. The orthogonal Partial Least Squares (OPLS) method is a recent modification of the PLS method that helps overcome pitfalls; as it separates systematic variation in X into two parts, one linearly related to Y and and one unrelated (orthogonal).(41, 49) Targeted metabolomics profiling is fundamentally different from chemometric approaches where the compounds in a given biofluid or tissue extract are identified and quantified by comparing the spectrum of interest to a library of reference spectra of pure compounds. The advantage of targeted metabolomics profiling is that it does not require the collection of an identical set, and is more amenable to human studies or studies that require less day to day monitoring. The main disadvantage is the relatively limited size of most current spectral libraries available for metabolite identification and interpretation. There are many free web based tools for data interpretation; some of which are summarized in **Table 0.2**.

Table 0.2: Web-based tools for targeted metabolomics data analysis.

Name of the web-based program	Website address	Characteristics	References
MetaboAnalyst	www.metaboanalyst.ca	Comprehensive analysis of quantitative metabolomics data, which includes data processing, data normalization, statistical analysis and high levels functional interpretations. Heat maps generated show fold changes of small metabolic compounds in different study groups which are involved in the cohort	(99)
MSEA, Metabolite Set Enrichment Analysis	www.msea.ca	Web-based tool to interpret patterns of human or mammalian metabolite concentration changes in a biologically meaningful context	(100)
MET-IDEA (Metabolomics Ion based Data Extraction Algorithm)	met-idea.software.informer.com	Capable of extracting semi-quantitative data from raw data files which allow rapid biological insight	(101)
MetaQuant	Bioinformatics.org/metaquant.	Java-based program for the automatic quantification of GC/MS-based metabolomics data,	(102)
XCMS	xcmsonline.scripps.edu	Allows users to upload easily and process liquid chromatography/mass spectrometry data for untargeted metabolomics	(103)
MZmine	https://omictools.com/mzmine-tool	Standalone Java application software, which implements solutions for several stages of data processing, including input file manipulation, spectral filtering, peak detection, chromatographic alignment, normalization, visualization and data export	(104)
MBRole	csbg.cnb.csi.es/mbrole/	Web server for carrying out over representation analysis of biological and chemical annotation in arbitrary set of metabolites	(105)

Together, with the results of the metabolic pathways analysis and other omics data, identified biomarkers are used to explain individual variations in disease and treatment responses. Also, individual or multiple biomarkers can be used for diagnostic purposes. Hence, metabolomics can be a valuable tool for precision medicine, for example; in the management of cancers like acute myeloid leukemia, the overexpression of 2-hydroxyglutarate has a therapeutic and multiple sclerosis.(106, 107) **Table 0.3**

1.5. Clinical Metabolomics towards Personalized Medicine

Personalized or precision medicine is a tool for disease prevention and management, which takes into consideration the individual's genomic variations, biochemical profile, environment, and lifestyle and aims at enabling advanced clinical decision-making. The introduction of President Obama's "Precision Medicine Initiative" in January 2015 has significantly popularized personalized medicine since then. As the percentage of patients for whom a drug is ineffective is quite significant in some groups such as cancer drugs (75%), Alzheimer's drugs (70%), personalized medicine is expected to help clinicians provide the most efficient management which requires personalized diagnosis and treatment.(106) In principle, the concept of personalized medicine originally focused on genotype guided prescription of drugs safely and efficiently. Empirical switching between drugs to find a desirable one with the satisfactory therapeutic response and least toxicity profile is not an ideal clinical practice. Thus, finding biomarkers that inform the understanding of disease pathophysiology should help in personalizing the diagnosis and management stratification. In addition, MS-based metabolomics has high potential to detect the molecular footprints of human disease, where ideally, the discovered biomarkers can be validated for use in routine clinical practice.

Table 0.3: Comparison between the analytical techniques in metabolomics (NMR, GC-MS and LC-MS)

Techniques	**NMR**	**GC-MS**	**LC-MS**
Target molecules	Amino acids, organic acids and sugars (water soluble)	Water soluble and some non-water soluble	Amino acids, organic acids and sugars (water soluble)
Types of samples	Bio-fluids, plants, bacterial, animal tissue extracts and food	Bio-fluids, plants, bacterial , animal tissue extracts and food	Bio-fluids, plants ,bacterial, animal tissue extracts and food
Minimum sample volume (µL)	100	30-50	10
Clinical examples	Amyotrophic lateral sclerosis and Parkinson disease(59), Multiple Sclerosis(61), Phenylketonuria(64), Systemic lupus erythematous(109), Juvenile idiopathic arthritis (69), Diabetes(47), Asthma(29)	Metabolites	Cystic fibrosis(42), Esophageal carcinoma(50), Diabetes(110), Oral, Breast and pancreatic cancer(48), Mild cognitive Impairment and Alzheimer disease(60) Snyder-Robinson syndrome(62), Pediatric chronic kidney disease(63)
Sample preparation time **(** min/20 samples)	30-120	120-360	30-60
Run time **(** min/sample)	20-90	30-60	2-30
Data analysis **(** min/sample)	30-60	30-60	2-30
Limit of detection(µM)	5.0	100.0	2.0
Number of detected metabolites	20-50	20-50	2-500
Overlapping metabolites	10-15	10-15	10-15
Cross checking (%)	10-30	10-30	10-30

Thus, there is a need for rigorous, standardized protocols, and pipelines for biomarkers discovery, analysis, validation, and reporting. Ultimately, these metabolic biomarkers may also lead to the development of novel therapeutics biomarkers that can be used to determine disease predisposition, such as *BRCA1* and *BRCA2* genes in breast cancer, diagnosis, prognosis or therapeutic responses. On the other hand, a diagnostic biomarker such as sweat chloride is used in combination with the clinical presentation to confirm a suspected diagnosis compatible with cystic fibrosis (surrogate). Oncotype Dx, a 21 mRNAs expression profile, is used clinically as a prognostic biomarker in patients with early stage, estrogen receptor positive breast cancer to predict recurrence risk as well as the likelihood of benefit from chemotherapy after breast cancer surgery. IL-17F, a predictive biomarker, could predict poor response to interferon-beta therapy in some patients with relapsing remitting multiple sclerosis.(106, 107)

1.5.1. Personalized medicine in cancer

Cancer is a major public health concern and a leading cause of death worldwide, accounting for 7.6 million deaths in 2008. Deaths from cancer are projected to rise to over 11 million by 2030. Since early diagnosis will improve the prognosis, the discovery of sensitive cancer-related biomarkers through a personalized approach has become a priority in cancer research and is used as a detection tool for therapeutic targeting of metabolic enzymes. The successful translation of such strategies to the treatment of cancer would allow earlier intervention to improve survival. For example, in patients with non small cell lung cancer, targeted drugs are available to treat these tumors that harbor EGFR and ALK mutations.(111) The central carbon metabolism varies between cancer and normal cells as described by Warburg in 1930, when he demonstrated that cancerous cells exhibit glucose fermentation (glycolysis) as an oxidation mechanism over oxidative phosphorylation even when enough oxygen is usually present.(112)This effect could result in a significant metabolic perturbation, which is tumor

specific. Several key metabolic enzymes might be secondarily affected by the alteration of oncogenes (i e, *RAS, PTEN, ERK*) and onco- transcription factors (i e, *HIF, p53, c-MYC*), which contribute to metabolic shifts as observed in cancer. An LC-MSMS-based metabolomics study showed that shift in blood lysophosphatidylcholine metabolism might drive tumorigenesis in breast, prostate, and colorectal cancer. These biomarkers were validated to show the clinical predictive value of cancer status with 69% sensitivity and 94% specificity.(53) Using LC/MS-Q-TOF, Bannur et al demonstrated differential expression of metabolites in patients with acute lymphoblastic leukemia undergoing treatment compared to those in relapse. Hence, these differences in metabolomic expression could be used to monitor disease progression, and potentially guide additional therapeutic interventions.(113) Slamon et al, first described the role of amplification of the oncogene ERBB2 (HER2), a cell surface tyrosine kinase non-ligand binding EGF receptor, in human breast and ovarian cancer.(114) Herceptin (Trastuzumab), an anti-ERBB2 (HER2) was developed to suppress the oncogenic effects of HER2 protein overexpression, which then served as a precision medicine, FDA approved diagnostic and therapeutic biomarker in HER2 positive metastatic breast cancers. However, Trastuzumab is not beneficial and may harm patients with cancers that do not overexpress HER2, as it is directed only to 30% of breast cancers with over expression of HER2. Tenori (et al), analyzed 579 serum samples from women with metastatic breast cancer being treated with paclitaxel plus either anti-HER2 treatment (lapatinib) or placebo. The metabolic profiles were compared with the time to progression, overall survival and treatment toxicity. The subgroup of patients with HER2 positive disease treated with paclitaxel plus lapatinib were found to be statistically significant compared to the other group, indicating the importance of the applicability of metabolomics in personalized response to medicine.(115)

Liesenfeld et al in 2013 published a literature review on metabolomics in cancer research, which included 106 studies reporting on 21 different types of cancer in seven different sample types. Alternations in metabolites in various types of cancer and standardization of data analysis showed that metabolomics could emerge as a tool for future applications in epidemiology and translational cancer research.(116) More recently, the introduction of iKnife, a metabolomics biomarker based device, has created a sensation in the field of cancer surgery.(117) This device combines an established technology "Electro- surgery," which was invented in the 1920s, and the new emerging technology "Rapid Evaporative Ionization Mass Spectrometry (REIMS)." This new technology promises to almost instantly identify and personalize the clinically relevant, tumor related metabolites present in human tissue by analyzing the smoke (heat generated from the current vaporized tissue) that is released during electro surgery. Once the REIMS technology is validated clinically, the "smoke based" metabolomics profiling of healthy and cancerous cells can instantly inform the surgeon whether the tissue is healthy or cancerous.

1.5.2. Personalized medicine in diabetes

Diabetes mellitus (DM) is a chronic disease characterized by hyperglycemia, caused by the autoimmune mediated failure of pancreatic β- cell function and resulting lack of insulin production (type 1 or insulin dependent DM) or by insulin resistance (type 2, non- insulin dependent DM). Fasting plasma glucose and hemoglobin A1c (HbA1c) along with oral glucose tolerance test are the traditional tests done to diagnose people with diabetes mellitus as well pre-diabetes. Since HbA1C levels reflect glycemic control over the previous several months, there has been ongoing interest in developing newer more precise disease specific biomarkers.(118, 119) Insulin resistance (IR), is a known early major risk marker for metabolic and cardiovascular disease and can precede dysglycemia in prediabetes and type 2 diabetes mellitus (T2DM) by some years. In contrast to the traditional glycated hemoglobin

(HbA1C), new non- glycemic (α-hydroxybutyrate, linoleoyl-glycerophosphocholine (LGPC), oleic acid) biomarkers, in combination with insulin, provide a novel and comprehensive measure of insulin resistance in a single assay. (Quantose IR™).(119, 120) Alpha hydroxybutyrate is positively correlated with insulin resistance and is indicative of early beta cell dysfunction, while L-GPC, is negatively correlated with IR shows impaired glucose tolerance and oleic acid is positively correlated with increasing lipolysis and IR.

Lastly, increase insulin level is characteristic of IR and is an independent risk factor for type 2 diabetes and cardiovascular disease. Quantose IR is a clinically validated fasting blood test for insulin resistance and pre-diabetes. The Quantose IR Score is based on the combined levels of these four biomarkers. Unlike the known T2DM related metabolomic biomarkers like branched chain amino acids, Wang-Sattler et al showed significant perturbations in glycine, lysophosphatidyl cholione (C18:2) and aceltycarnitine levels in pre-diabetic individuals with impaired glucose tolerance when compared to those with normal glucose tolerance. Metabolite protein interaction networks analysis linked these three biomarkers to seven known T2DM related genes (*PPARG, TCF7L2, HNF1A, GCK, IGF1, IRS1,* and *IDE*) through related enzymes or proteins and was confirmed by demonstrating altered transcription levels.(121) Suhre et al showed that perturbations of metabolic pathways linked to kidney dysfunction (3-indoxyl sulfate), lipid metabolism (glycerophospholipids, free fatty acids) and interaction with gut microflora (bile acids) might detect diabetes related complications under sub-clinical conditions.(47)

Patients with T2DM enjoy the clinical availability of several classes of antidiabetes drugs including sulphonylureas, dipeptidyl peptidase-4 inhibitors, glucagon-like peptide-1 receptor agonists, thiazolidinediones, sodium- glucose cotransporter-2 inhibitors, and biguanides (metformin). Given the complex genetics underlying the etiology of T2DM, it is not

surprising to note varying degrees of benefit and harm of these drugs in such patient population. Therefore, application of a personalized medicine approach based on recently discovered pharmacogenetics data is becoming increasingly useful. This is most evident in patients with sulphonylurea sensitivity, with HNF1A maturity-onset diabetes of the young (MODY) and patients with T2DM in combination with reduced function alleles at CYP2C9. This resulted in reduced metabolism of sulphonylureas, therefore increasing the risk of related side effects and severe metformin intolerance associated with reduced function organic cation transporter 1 (OCT1) variants, exacerbated by drugs that also inhibit OCT1.

1.5.3.Inborn errors of metabolism

Inborn Errors of metabolism (IEM) are inherited disorders typically caused by recessive mutations in the genes encoding metabolic enzymes. There are hundreds of IEMs and they have wide clinical spectrum. In a proof of concept study, untargeted metabolomic profiling was used efficiently in biomarker identification in patients with disorders of propionate metabolism. Out of 3500 segregated metabolites, propionyl carnitine was identified as distinguishing biomarker between methylmalonic and propionic acidemias. Perturbations in γ-butyrobetaine (despite normal free carnitine), isovalerylcarnitine and unsaturated acylcarnitines were associated with these two diseases and contributed to their improved understanding, disease diagnosis, and clinical patient evaluation.(72) Recently, phenylketonuria pathogenesis was studied using metabolomics approaches, where patient's urine samples were profiled using GC-MS and NMR. Through multivariate and univariate statistical analyses, additional pathophysiological perturbations were found in arginine, proline, alanine, aspartate, and glutamate metabolism pathways.(123)

Miller et al, studied 120 individual plasma samples and demonstrated the possibility of prospective screening of IEMs that might otherwise require ordering multiple confirmatory

tests. This semi quantitative method detects 900 compounds, including 500 human analytes in a single human plasma sample.(124) These and other related studies formed the basis for the commercial launching of the two comprehensive targeted metabolomics based screens: Global Metabolomic Assisted Pathway Screen (GMAPS™) and MetaIMD™.(85) (84)

In summary, the current clinical practice approach works for the majority of the population, but misdiagnosis, symptoms relapse, and unfortunate side effects that occur in some people limit the clinical efficacy in disease control. By comparing the metabolomic profiles of two or more disease phenotypes, biomarkers related to the disease specific perturbations can be investigated to develop a personalized approach used in treating and monitoring disease progression.(125) Since metabolomics can be performed on various biological matrices including CSF, urine, plasma, and sweat, standardized procedures are critical to rule out external interferences originating from those matrices. The time gap between sample collection and sample preparation, if not controlled, can result in significant variability in the metabolomics profile. Furthermore, improper storage and repeated freeze-thawing cycles hurt the quality of the obtained data.

Dealing with the simultaneous analysis of different classes of metabolites, the chemical complexity, and molecular heterogeneity can pose technical challenges in analyzing them in a single assay. Choosing a single sample preparation method that provides both adequate sample clean up and universal extraction properties is also challenging.(126) Studies have shown that it is not practical to utilize a single stationary phase for the separation of a wide range of metabolites with different polarities.(127) Furthermore, unknown exogenous metabolites can be observed in the metabolome, where uncertainty can be avoided by generating larger databases to enable better separation from “normal” routine samples.(128) In GC-MS, the type and amount of derivatization reagent need to be optimized carefully

otherwise it may introduce additional interfering compounds, produce unexpected derivatives and by-products, and may even damage the column.

Metabolomics data are vast and complex; however, the use of multivariate analytical techniques for data processing and availability of high-quality analysis software has made the data less complex. Improved methods for metabolomics data analysis are regularly published, making it difficult to choose the most appropriate approach. Nonetheless, metabolomics is still somewhat lagging behind other "omics" due to limited technical development, database challenges, training, and costs. More collaborations and exchanges with scientists in other disciplines need to be encouraged. Metabolomics has influenced pharmacological research to a great extent and made personalized health care increasingly possible. In silico models are increasingly used to elucidate the effect of drugs on metabolic phenotypes before moving on to in vivo testing. Gut microbiota associated with specific diseases can be better understood with the help of metabolomics, which can contribute to drug development.(129) Despite all these challenges, metabolomics along with the other "omics" facilitates the development of more efficient and precise biomarkers important for the understanding of disease progression and drug development.

Van der Greef et al, recommend a one case clinical trial approach, wherein patients are evaluated by a challenge test approach like the oral glucose tolerance test (OGTT) routinely done to test for early onset of T2D, and is evaluated against an extensive database of information on environment, lifestyle, nutrition, psychological recommendations and also genotype/phenotype information related to health and disease. A Metabolomics study of the phenotype along with the challenge test will help to diagnose the disease quite earlier and also improve the health care system.(130) Due to the considerable improvements in health care delivery for individuals with acute health conditions, the incidence of chronic diseases is

rising sharply. It is estimated that by 2020, chronic diseases are likely to account for three-quarters of deaths worldwide. Hence, there is a need to develop new effective approaches for personalized diagnostics with impact on therapeutic intervention and monitoring. Metabolomics contributes significantly to the characterization of various disease phenotypes and the identification of personalized metabolic features that can predict response to therapeutic interventions.

The contribution of metabolomics to personalized health care and biomedical sciences, is immeasurable and has enabled the discovery of biomarkers specific enough to distinguish patients in various health states from healthy subjects. Differentiating DOCK8 deficient patients from those with atopic diseases at an early stage is significant in a child's development, as treatment modalities differ considerably. To date, no metabolomics data is available to differentiate between DOCK8-deficient and AD patients, which present with similar clinical profiles. Therefore using metabolomics to understand the metabolic fingerprinting in this study cohort may help investigate the inflammatory responses that develop during disease pathogenesis.

1.6. Summary and Research synopsis of the Thesis

In this chapter, a detailed review of current metabolomics literature has been highlighted, to understand the metabolomics strategies and analytical approaches that focus on the discovery of biomarkers. The literature was derived from metabolomics biomarker studies in the field of cancer, diabetes, obesity, inborn errors of metabolism and personalized medicine.
The work presented in this PhD thesis provides a comprehensive biomarker discovery study in DOCK-8 deficient and AD patients. Particular emphasis was laid on building a comprehensive metabolomics panel and in discovery of cytokine and metabolomics

biomarkers. Since these patients have food allergies in common, food sensitization patterns were also studied.

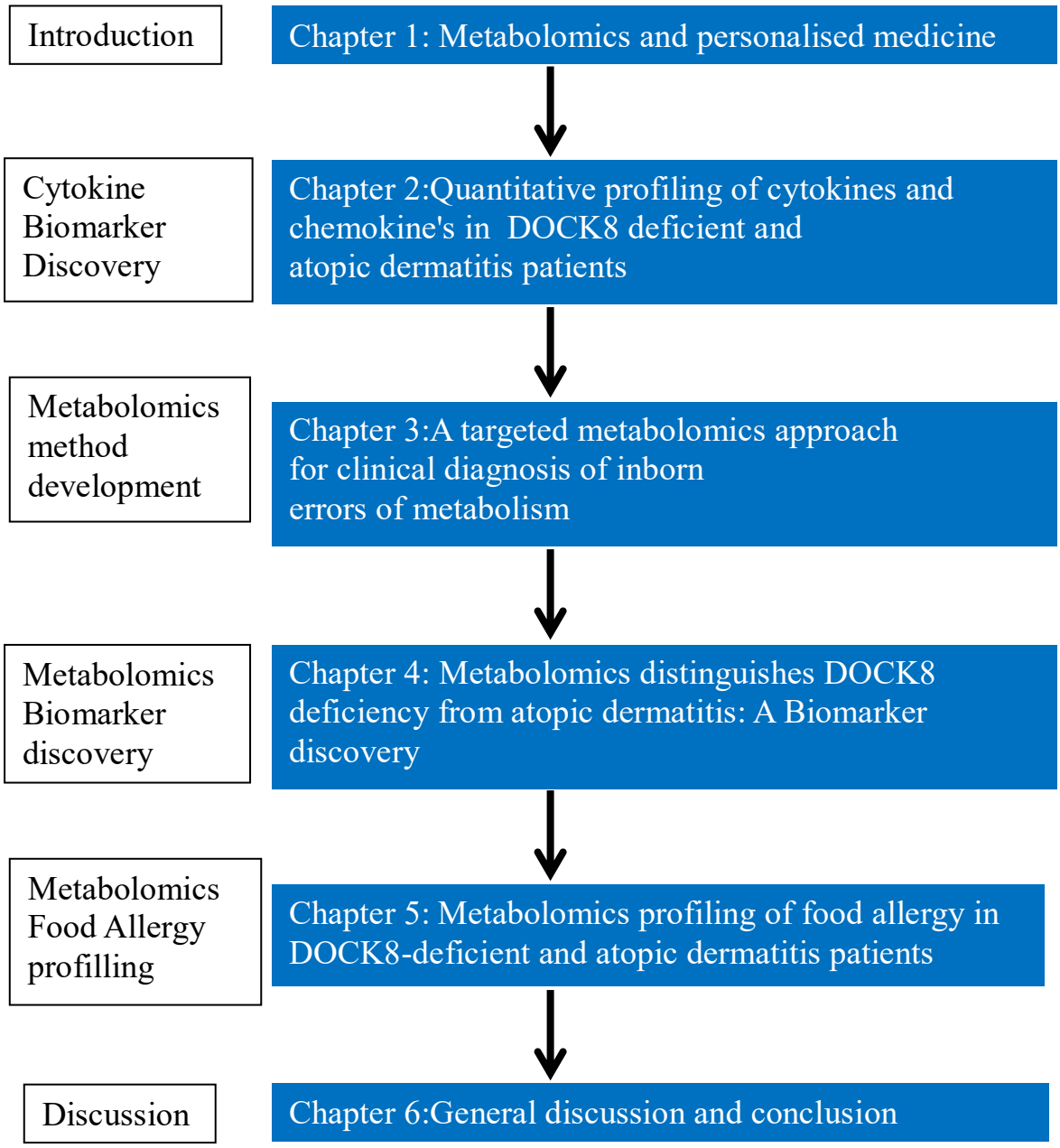
In **Chapter 2**, an extensive Quantitative profiling of Cytokines and Chemokine was generated and the comprehensive cytokine profile in HIES patients revealed distinctive biomarkers that differentiate between the DOCK8 deficient and AD patients. The unique expression profile of various inflammatory cytokines in patients with DOCK8 deficiency vs. atopic dermatitis likely reflects disease-specific perturbations in multiple cellular processes and pathways leading to a predisposition to infections and allergies seen in these patients.

Chapter 3, details a robust comprehensive metabolomics method using tandem mass spectrometry for identification of the biomarker metabolites involved in DOCK-8 deficient and AD patients.

In **Chapter 4**, in-depth metabolomics technologies including CIL LC-MS targeting the amine/phenol sub-metabolomes was used to study and explore differentially expressed biomarkers in DOCK8-deficient and AD patient groups. These biomarkers potentially reflect upon the disease pathogenesis and may contribute towards improved disease monitoring and ultimately novel clinical interventions.

In the final **Chapter 5,** Sensitization patterns and cross reactivity of food and aero-allergens was analysed using the ImmunoCAP ISAC in correlation with the metabolomics and cytokine profiles from chapter 2 and 4. The distinct metabolic fingerprinting in food allergy in our cohort, reflects upon the inflammatory responses that develop during DOCK8 pathogenesis.

The work presented in this thesis provides an important contribution towards the discovery of biomarkers in DOCK8-deficient and atopic dermatitis patients.



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CHAPTER 2: QUANTITATIVE PROFILING OF CYTOKINES AND CHEMOKINES, IN DOCK8-DEFICIENT AND ATOPIC DERMATITIS PATIENTS.

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2.1 Introduction

Hyper-IgE (IgE) syndromes [HIES] are primary immunodeficiency disorders, characterized by elevated IgE levels, recurrent staphylococcal skin abscesses, eczema, and pulmonary infections.(1) They are genetically heterogeneous inherited syndromes, first described by Davis et al.(1966) and linked mainly to mutations in signal transducer activator of transcription 3 (STAT3) or dedicator of cytokinesis 8 (DOCK8), either in an autosomal dominant or recessive pattern, respectively.(2-4) Recently, additional mutations in phosphoglucomutase 3 (PGM3), involved in the glycosylation pathway, ZNF341 (5, 6) and IL6ST have been identified in the autosomal recessive forms of HIES.(7, 8) In contrast to STAT3 mutations, DOCK8-deficient patients have severe T-cell dysfunction associated with severe inflammation and allergic manifestations.(9, 10) Common pathogens include herpes simplex virus (HSV), human papillomavirus (HPV), molluscum contagiosum virus (MCV) and varicella zoster virus (VZV). Susceptibility to recurrent sinopulmonary infections is also caused by a wide variety of pathogens including streptococcus pneumoniae, haemophilus influenzae, pneumocystis jirovecii histoplasma capsulatum, and legionella pneumophila. CD8 T cells play an important role in immunity, (11) Randall et.al (2011) showed that DOCK8 plays a critical and intrinsic role in peripheral CD8 T cell survival and function.(12) Most of DOCK8 patients have low absolute counts of both CD4 and CD8 T cells, indicating a T cell immunity defect.Cancers related to cutaneous viral infections and T-cell lymphoma had been described in DOCK8 deficiency, which is thought to be related to impaired CD8 T cell functions suggesting that DOCK8 has a tumor suppressor function.(9) DOCK8 is mainly expressed in cells of the immune system and controls many cellular processes including migration, phagocytosis, and adhesions.(10) A study by Su Hu et al. emphasized the regulatory role of DOCK8 on lymphocytes, where DOCK8 is essential for the activity of these immune cells, and its absence leading to susceptibility to infection.(8)

DOCK8 is one of eleven members of the DOCK 180 superfamily, (14) and has a typical guanine nucleotide exchange factor (GEF) for small G proteins, of Rho family such as Rac and Cdc42. DOCK8 mainly has two related conserved protein domains DHR1 and DHR2, wherein the DHR1 domain binds to phosphatidylinositol-3, 4, 5-triphosphate (PtdIns 3,4,5)P3 and mediates the recruitment of DOCK proteins to the plasma membrane. (15, 16) DOCK8 acts as an adapter connecting Toll like receptor 9(TLR9) to the signalling cascade of B cell antigen receptor (BCR). DOCK8 also connects TLR9 signalling to the activation of STAT3 and activation of TLR9 by its ligand CpG induces the formation and stabilization of a trimeric signalling complexes consisting of MYD88, PYK2 and DOCK8. This leads to the activation of src kinases which together with syk phosphorylate, activates STAT3, whose target genes are linked to the survival of B cells and production of immunoglobulin. This pathway indicates that DOCK8 is essential for TLR9-driven B cell proliferation and immunoglobulin production. (17, 18)

DOCK8 patients are prone to autoimmune diseases; autoimmunity has been linked to defects in B cell tolerance sites involved in the elimination of auto reactive B cells. Janssen et.al (2014) showed that DOCK8 plays a crucial role in Treg cell homeostasis function and peripheral B cell tolerance.(19) Natural killer T(NKT) cells are a group of immunoregulatory T lymphocytes that play a important role in many diseases including viral infections, cancer, autoimmunity and allergy.(20) DOCK8 deficiency results in weak development of NK T cells thereby affecting their formation, development and survival, resulting in susceptibility to infections and malignancy.(21, 22)

Atopic dermatitis (AD) is a pervasive pediatric, chronic, highly pruritic inflammatory skin disorder, commonly associated with high IgE level. Eczematous skin lesions and pruritus are common overlapping clinical features in all subtypes of HIES and AD suggesting immune dysregulation. Through Endothelial Per-Arnt-Sim (PAS) domain protein 1 (EPAS1)

dependent activation of the IL-31 promoter, CD4+ T cells from DOCK8-deficient mice were recently shown to produce large quantities of IL-31, a significant pruritogen associated with AD.(11)

It is speculated that the severity of infection and the skin hypersensitivity could be due to an altered cytokine production pattern in DOCK8 deficient and AD patients. The clinical management of patients with moderate-severe AD and DOCK8 deficiency can be challenging and quite complex, as they share many clinical features in common. A study by Janssen et.al (2014) has distinguished DOCK8 deficiency from AD using flow cytometry. CD3 and CD4 lymphopenia and decreased CD8 T cells coupled with decreased memory B cells is strongly suggestive of DOCK8 deficiency rather than AD in a patient with severe eczema.(24) Patient and family education, avoidance of known contact, food and aero-allergens, controlling pruritis (using antihistamines) and proper skin hygiene and hydration using emollients and moisturizers are important first steps of treatment. Topical therapy includes using corticosteroids of varying potencies, calcineurin inhibitors, phosphodiesterase 4 (PDE4) inhibitor. (crisaborole)

Acute exacerbations of chronic AD may require systemic corticosteroids. Phototherapy as well as immune suppression (such as oral cyclosporin) is more frequently and regularly used in adults compared to children. More recently, systemic (subcutaneous) immunomodulation using dupilumab, an (IgG4) anti-IL-4 and IL-13 receptor blocker, became available as an alternative option for patients with very difficult to control moderate to severe atopic dermatitis. Viral and bacterial cutaneous infections also require special attention and prompt management. As DOCK8 deficiency is a complex systemic disorder, these patients require additional supportive therapies and ultimately a definitive curative treatment with hematopoietic stem cell therapy (HSCT). Adequate nutritional support (to prevent failure to

thrive and secondary nutritional deficiencies), pulmonary and liver function assessments and monitoring, prophylactic antibacterial, antiviral as well as antifungal (cryptosporidial infections) and immunoglobulin prophylaxis are frequently required. Due to the high morbidity and mortality associated with DOCK8 deficiency, and as several clinical reports have demonstrated high survival, robust cellular and humoral immune reconstitution and improvements in both infections and atopy post- transplant, HSCT became the preferred definitive therapy for this and many other disorders.(25-30)

Cytokines and their receptors play an essential role in the development, homeostasis as well as pathogenesis of tumors in HIES. High levels of IgE seen in these patients signal cytokine dysregulation and an imbalance in Th1/Th2 cytokine profile contributing toward the pathogenesis of the disease.(31) Altered or dysregulated cytokine expression leads to pathologies such as inflammation, tumorigenesis, and autoimmunity. Patients with DOCK8 deficiency have increased Th2 cells (expression of IL-4, IL-5, IL-10, IL-13), which indicate its role in T-cell regulation of allergic disease.(32) IL-31 produced by Th2 cells is involved in promoting dermatitis and epithelial responses that characterize allergic and nonallergic diseases.(33) Renner et al. (2005) reported that despite the imbalance in T cell responses in HIES patients there is no defect in toll-like receptor signalling, (34, 35) These studies have led to the hypothesis that HIES might be a disease of impaired inflammatory responses rather than a disease of pathologic IgE production. Elevated IgE levels, eczema, asthma, food, and environmental allergies are common to HIES and AD, although warts, severe bacterial and viral infections, and pneumonia are exclusively seen in DOCK8-deficient patients.(3, 35, 36) Therefore, measurement of immune-related proteins in serum may help in characterizing the local inflammatory responses that develop during HIES pathogenesis. Differentiating DOCK8-deficient patients from those with atopic diseases at an early stage is significant in a child's development, as treatment modalities differ considerably.

2.2 Aims

The aims of this study were as follows

1) Comprehensive cytokine/chemokine profiling, in patients with DOCK8 deficiencies and Atopic dermatitis.
2) To analyze the cytokine data using metabolomics to find distinct cytokine biomarkers, to differentiate between DOCK8 deficiencies and Atopic dermatitis patients.

2.3 Methods

2.3.1 Study Design and ethics

Symptomatic patients with confirmed pathogenic DOCK8 mutations (n = 10), moderate to severe AD (n = 9) diagnosed based on the Hanifin and Rajka clinical criteria (37) and healthy Controls (ctrls) (n = 15) were enrolled in this study (**Table 0.1)**. All recruited subjects attended the Allergy/Immunology Clinics at King Faisal Specialist Hospital and Research Centre (KFSHRC). Samples from the study participants were collected after signed informed consent was provided. Clinical and laboratory findings, as well as a comprehensive baseline questionnaire, were completed for each patient by the recruiting clinician. The consent form and baseline questionnaire were reviewed and approved by the Institutional Review Board's (IRB) ethics committee at KFSHRC (RAC No. 2160015). The Severity Scoring of AD (SCORAD) and Visual analogue scale (VAS) pruritus scores were determined for the DOCK8-deficient and AD groups.(38)

2.3.2 Cell lines

The lymphoblastoid cell lines were established by transformation of peripheral blood mononuclear cells (PBMCs) with Epstein-Barr virus as per standard protocols. Blood collected in an anti-coagulated sodium heparin tube was processed for lymphocyte separation, by adding Ficoll-paque, (GE Healthcare, Sigma Aldrich) followed by centrifugation at 3000 revolution per minute (RPM) for 20 minutes. Lymphocytes were transferred and washed with PBS and centrifuged at 3000 RPM for 10 minutes. Lymphocytes were re-suspended in Roswell Park Memorial Institute (RPMI) 1640 media supplemented with 15% fetal bovine serum (FBS), 50 units/mL penicillin, a mixture of 1% glutamine. (Gibco, Life Technologies). This was followed by addition of 0.5ml of Epstein Barr Virus (EBV) and 5µl of phytohaemagglutinin (PHA) to the re-suspension media. The lymphocytes were incubated at 37ºC in 5% CO2-humidified tissue culture incubator. The lymphocytes

were re-fed and cells were harvested during the logarithmic growth phase. After the incubation period, the cell media was centrifuged and supernatants were separated and stored at -80^{0}C.

2.3.3 Multiplex cytokine/chemokines analysis

The human magnetic bead panel array was used to quantify the cytokine/chemokine levels in patient's sera. The MILLIPLEX MAP kit (Millipore) was used according to the manufacturer's instructions. Color code microspheres coated with a specific capture antibody were incubated with 100 uL of serum. The phycoerythrin-conjugated detection was used to form sandwich complexes, and the fluorescence data was acquired on the Luminex appliance, FLEXMAP 3D where one laser excites the phycoerythrin and measures the analytes whereas the other laser detects the fluorophores inside the bead and measures its expression in the sample. The targeted cytokines (n= 39) are listed in supplementary **Table A1.1.1**. Serum samples were assayed in duplicates, and analyte concentrations were calculated by constructing a standard curve to convert the optical density values into concentrations (pg/ml) using the Milliplex Analyst software, version 5.1 (Vigene Tech).

Table 0.1: Laboratory findings in DOCK8 and Atopic Dermatitis Patients

Diagnosis		Mutation	Age (Y)	Sex	IgE Levels (KU/L)	Hgb 110-160g/L	Hct 0.370-0.520L/L	WBC 10^9/L	Eosinophils 10^9/L	CD4/CD8 Ratio	VAS	SCORAD
DOCK8	P1	NM_203447.3:c.[2606-1G>A; c.405_827del]	21	F	25000	114	0.341	9.2	24.8	2.1	15	68.7
	P2	NM_203447.3:c.[2606-1G>A; c.405_827del]	16	M	9140	86	0.264	3.72	35	0.8	13	67
	P3	NM_203447.3:c.5625T>G:p.Y1875*	16	F	1690	121	0.36	4.63	50.3	1	14	59.8
	P4	NM_203447:c.827+6T>C	16	F	11240	128	0.395	4.9	11.4	0.73	15	77.4
	P5	NM_203447.3:c.5962_6068del (exon 46)	7	M	10170	103	0.312	9.93	26.4	0.7	17	79.5
	P6	NM_001193536:c.3949+1G>T	14	M	15550	178	0.549	4.46	11.7	1.3	15	62.5
	P7	NM_001193536:c.1593+1G>T	4	M	86940	85	0.29	24.9	31.4	9	17	70.6
	P8	NM_001193536:c.1593+1G>T	9	F	44630	103	0.354	12.49	34.8	8.1	9	58.2
	P9	NM_001190458:c.1905_1905+1delGG	8	F	26340	99	0.328	21.42	35.1	3.2	14	58.8
	P10	NM_203447.3:c.5625T>G:p.Y1875*	21	M	265	119	0.386	9.7	12	1.1	12	70.1
Average±SEM			**13.2±5.9**	**5/5 (M/F)**	**19817.30±4772.6**	**113.6±8.4**	**0.357±0.02**	**10.53±2.3**	**27.29±4.02**	**2.8±0.99**	**14.1±0.75**	**67.26 ±2.37**
ATOPIC DERMATITIS	P1	Negative DOCK8 mutation	6	M	6540	124	0.368	10.6	16.5	0.9	16	64.4
	P2		15	M	16500	142	0.419	3.72	15.6	1.4	14	71.3
	P3		10	M	1612	102	0.32	8.01	13.9	1.2	16	73.3
	P4		3	M	839	124	0.392	6.59	6.7	_	13	56.1
	P5		8	M	1221	119	0.388	5.09	13	1.5	9	40.8
	P6		13	M	659	110	0.371	5.15	13	1.2	10	54.9
	P7		15	F	1387	115	0.364	9.63	12.3	1.4	15	75
	P8		16	M	365	133	0.372	2.71	7.9	2.3	14	65.1
	P9		12	M	4587	127	0.417	9.2	5.1	1.5	16	70.6
Average±SEM			**10.8±1.4**	**8/1 (M/F)**	**5288.20±1736.3**	**121.7±4**	**0.379±0.01**	**6.74±0.92**	**27.29±1.3**	**1.43±0.14**	**13.6±0.86**	**63.5 ± 3.702**

2.3.4 IL-31 measurement using ELISA

An ELISA kit (R&D Systems Minneapolis, USA) was used to quantify IL-31 in human sera. The samples were incubated with an anti-human IL-31 primary antibody and then with the biotinylated secondary antibody. A streptavidin-labeled horseradish peroxidase (HRP) was coupled to the sandwich complex for color development. Fluorescence intensity was measured at 450 nm and 570 nm wavelengths of excitation and emission, respectively, using Varioskan Flash multimode reader (Thermo Fisher Scientific Pandan crescent, Singapore).

2.3.5 Immunoblotting

Cells were centrifuged at 500 RPM for 5 min at 4ºC in ice cold phosphate buffered saline (PBS). After removing the supernatant, cells were lysed in Radio Immuno Precipitation Assay (RIPA) buffer and the cell pellet were allowed to agitate in a micro-centrifuge tube for 30 min at 4°C. The tubes were centrifuged at 13000 RPM for 30 minutes and the supernatant containing the protein was collected into a tube and protein was quantified using a spectrophotometer. Thirty micrograms of protein was loaded separated by SDS-PAGE (National Diagnostics, Atlanta, USA). for separation. The protein bands were transferred onto a Poly vinylidene fluoride (PVDF) membrane (GE Healthcare Waukesha, WI, USA) and to prevent the antibodies from binding to the membrane non-specifically, nonfat dried diluted milk solution was used. The membrane was incubated with HRP-conjugated antibodies, anti-DOCK8 (Santa Cruz Biotechnology Dallas, TX, USA) and mouse anti-beta actin (Abcam Cambridge, MA, USA). The complex was detected using the SuperSignal West Pico Chemiluminescent (Thermo Fisher Scientific) and visualized on an ImageQuant LAS 4000 (GE Healthcare Life Sciences).

2.3.6 DOCK8 mutation analysis

DOCK8 mutations were confirmed by Sanger sequencing of gDNA isolated from peripheral blood.(35)

2.3.7 Data and statistical analysis

The cytokines/chemokines global data analysis was performed using MetaboAnalyst version 3.0 (McGill University). The raw data were normalized to the sample total median to ensure all samples were normally distributed. To visualize the difference among the study groups and make individual features more comparable, data were log transformed, and Pareto scaled, respectively. As the data were Gaussian distributed, the unpaired two-tailed Student's t test was used for binary comparison between any two study groups, where the significance levels for cytokines data were considered at an false discovery rate (FDR) corrected p-value<0.05, and values were presented as mean ± SEM. The targeted statistical analysis of multiple study groups (data in Figures 1 and 2) was carried out using GraphPad Prism (version 6.0, GraphPad software LA Jolla, CA). Analysis of variance (ANOVA) was performed by choosing column mean type analysis. Post hoc Tukey's method of analysis was used for more binary analysis and information such as adjusted p-values for each comparison. These differences between the groups contributed to the significance of an ANOVA test. In all experiments, a calculated p-value<0.05 was considered to be statistically significant.

2.4 Results

2.4.1 Clinical profile of HIES patient

DOCK8 deficiency and AD patients were recruited through the allergy and immunology clinics at KFSHRC. In DOCK8-deficient patients group, 50% were females and the mean age

was 13.2±5.9 years, whereas for the AD group (8 males and 1 female) the mean age was 10, and for the Ctrl group 23± 1.03 years (8M/7F). Eosinophilia was noticeable in both groups, whereas the median WBCs were 1.93±0.6 10^12/L) and 11.29±2.44 (10^9/L), whereas the Hgb counts were 113.6±8.4 and 121.7±4 (110-160g/L) respectively.The Hct median were almost same in both the cohort: 0.356±0.02 and 0.379±0.01 (0.370-0.520 L/L) respectively. The median of the CD4/CD8 ratio in DOCK8 patients was 2.8±0.99, compared to 1.43±0.14 in the AD. **(Table 0.1).** DOCK8 mutations were confirmed by standard Sanger sequencing of peripheral blood gDNA. Dermatitis and food allergies were the most common clinical manifestations in both groups, followed by bronchial asthma. Staphylococcus infections and pneumonia were the most common clinical features seen in the DOCK8 patients, while four patients presented with thrush candidiasis and warts. **(Table 0.2)** In conjunction with severe skin infections, itching, and rashes, DOCK8 patients (50%) developed cutaneous abscesses. In addition, four DOCK8-deficient patients acquired viral infections caused by molluscum contagiosum, and two had sclerosing cholangitis, whereas AD patients had fungal infections of the scalp (22%) and rhinitis (50%). Furthermore, three AD patients developed ear and chest infections **(Table 0.2**) sinusitis was seen in four DOCK8-deficient patients. Owing to recurrent infections, six DOCK8-deficient patients showed retardation in their physical growth. Additional features of DOCK8 deficiency include cataract and seizures in two patients each, congestive heart failure in one patient and one who developed fatal Hodgkin lymphoma. Both cohorts demonstrated elevated serum IgE levels compared to Ctrls. The IgE levels were significantly higher in DOCK8 patients (p-value<0.05) than AD ranging from 5-500 KU/L (IU/ml) **(Error! Reference source not found.A**) In DOCK8, splicing mutations were t he most common (70%), followed by stop gain (20%) and deletion **(Error! Reference source not found.B**) Mutations in DOCK8 were excluded in all AD patients except one. Immunoblot based DOCK8 expression analysis of cell line extracts using an N-terminus specific antibody

(Error! Reference source not found.C) showed near complete loss of DOCK8 protein e xpression in all tested patients (n = 7) regardless of their mutation type. In three patients (P7, 8, 9), immunoblot analysis could not be performed due to inadequate sample.

Table 0.2: Clinical manifestations in DOCK8 and AD patients

Findings	DOCK8 (n=10) (%)	AD (n=9) (%)
Dry skin/itching	100	100
Atopic dermatitis/eczema	100	100
Food allergies	100	100
Pneumonia	100	0
Staphylococcal infection	100	11
Bronchial asthma	90	44
Bronchiectasis	50	0
Molluscum contagiosum	40	0
Sclerosing cholangitis	20	0
Thrush candidiasis	40	0
Warts	40	0
Abscesses	50	0
Otitis media	30	0
Sinusitis	40	0
Rhinitis	0	50
Hodgkin lymphoma	9	0
Failure to thrive	60	0
Seizures	20	0
Cataract	20	0
Congestive heart failure	10	0
Deceased (Hodgkin lymphoma)	10	0

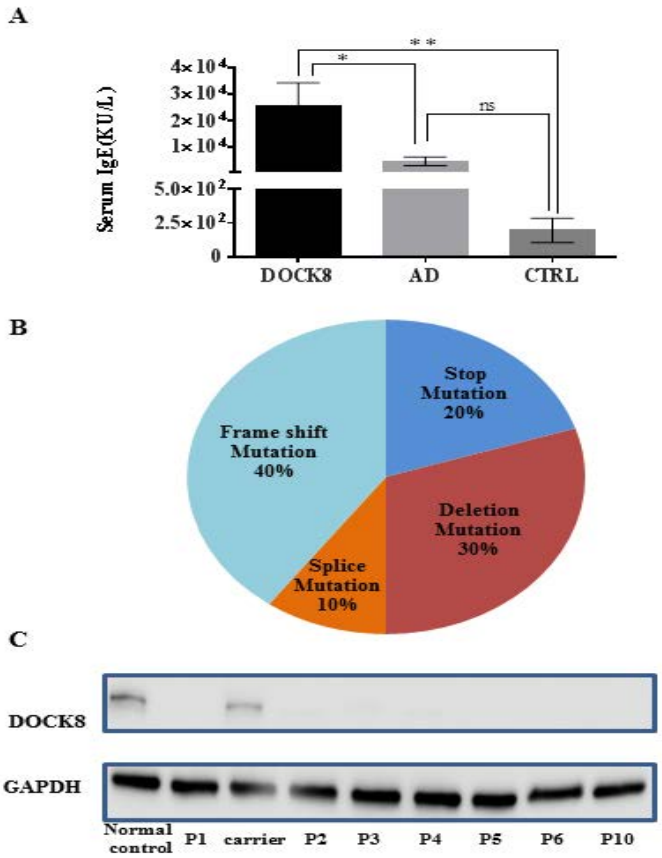

Figure 0.1: DOCK8 mutations and protein expression.**(A)** Serum IgE levels in patients with DOCK8 deficiency, atopic dermatitis (AD) and healthy controls (Ctrl) measured at collection point (One way ANOVA, Post hoc Tukey's * p-value <0.05, ** p-value <0.001). **(B)** Distribution of DOCK8 gene mutations in HIES patients with DOCK8 deficiency**. (C)** Western blot analysis of DOCK8 protein in human lymphoblastoid cells of patients with DOCK8 deficiency (P1-P6, and P10)

2.4.2 Changes in Serum cytokines

In this study, a full analysis of the 39 analytes revealed only seven that were differentially expressed among the three comparative groups at p-value <0.05.(**Table 0.3)** As shown in

A , CXCL10 was up-regulated in DOCK8-deficient patients compared to AD and Ctrl groups (P-value <0.001). TNF-A, GCSF and MDC were significantly upregulated only in DOCK8-deficient patients when compared to ctrl. **(**

B, C, E) CX3CL1 was significantly downregulated in DOCK8-deficient patients (p-value <0.05) compared to Ctrl groups **(**

D), whereas EGF was downregulated in AD (p-value <0.05) and not significant when DOCK8 group was compared to Ctrl. **(**

F) However, IL-31 was significantly upregulated in both patient groups compared to ctrls **(**

G).

2.4.3 Biomarker evaluation

For evaluating these cytokines for potential biomarker discovery, the data were combined and globally analyzed using MetaboAnalyst software version 3.0 (http://www.metaboanalyst.ca).

The raw data were normalized, transformed, and scaled by a median, log, and Pareto, respectively, to make sure all the data are visualized under Gaussian distribution. For paired analysis, a combination of the t test and fold change analyses is represented in a volcano plot. (**Figure A2.1**). The comparison between DOCK8-deficient and Ctrl groups was demonstrated in a orthogonal projections to latent structures discriminant analysis (OPLS-DA)

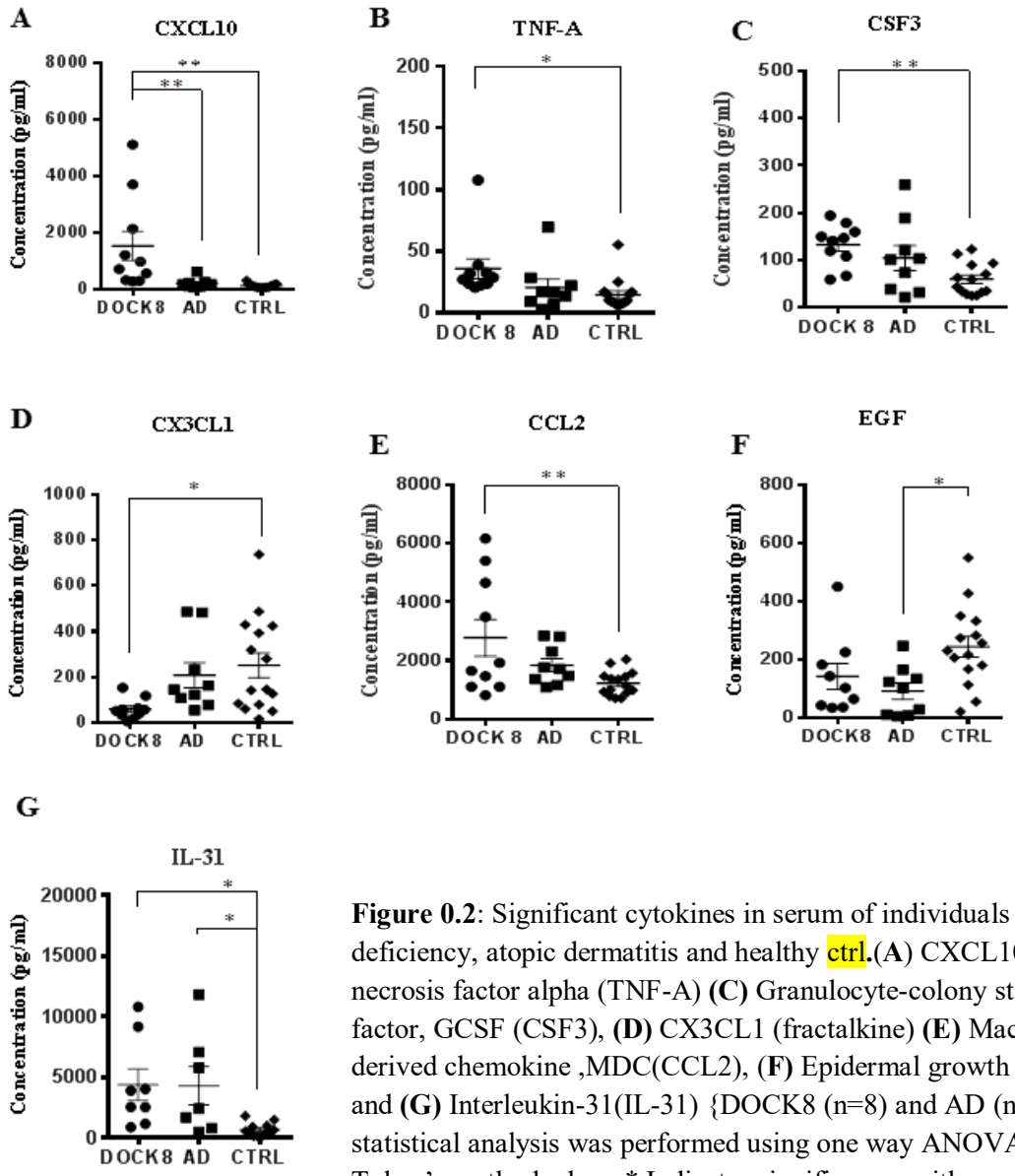

Figure 0.2: Significant cytokines in serum of individuals with DOCK8 deficiency, atopic dermatitis and healthy ctrl.(**A**) CXCL10**, (B)** Tumor necrosis factor alpha (TNF-A) **(C)** Granulocyte-colony stimulating factor, GCSF (CSF3), **(D)** CX3CL1 (fractalkine) **(E)** Macrophage-derived chemokine ,MDC(CCL2), **(F)** Epidermal growth factor (EGF) and **(G)** Interleukin-31(IL-31) {DOCK8 (n=8) and AD (n=7). The statistical analysis was performed using one way ANOVA, post hoc Tukey's,method where * Indicates significance with p-value <0.05, ** p-value <0.001, and otherwise are not significant (ns)}.

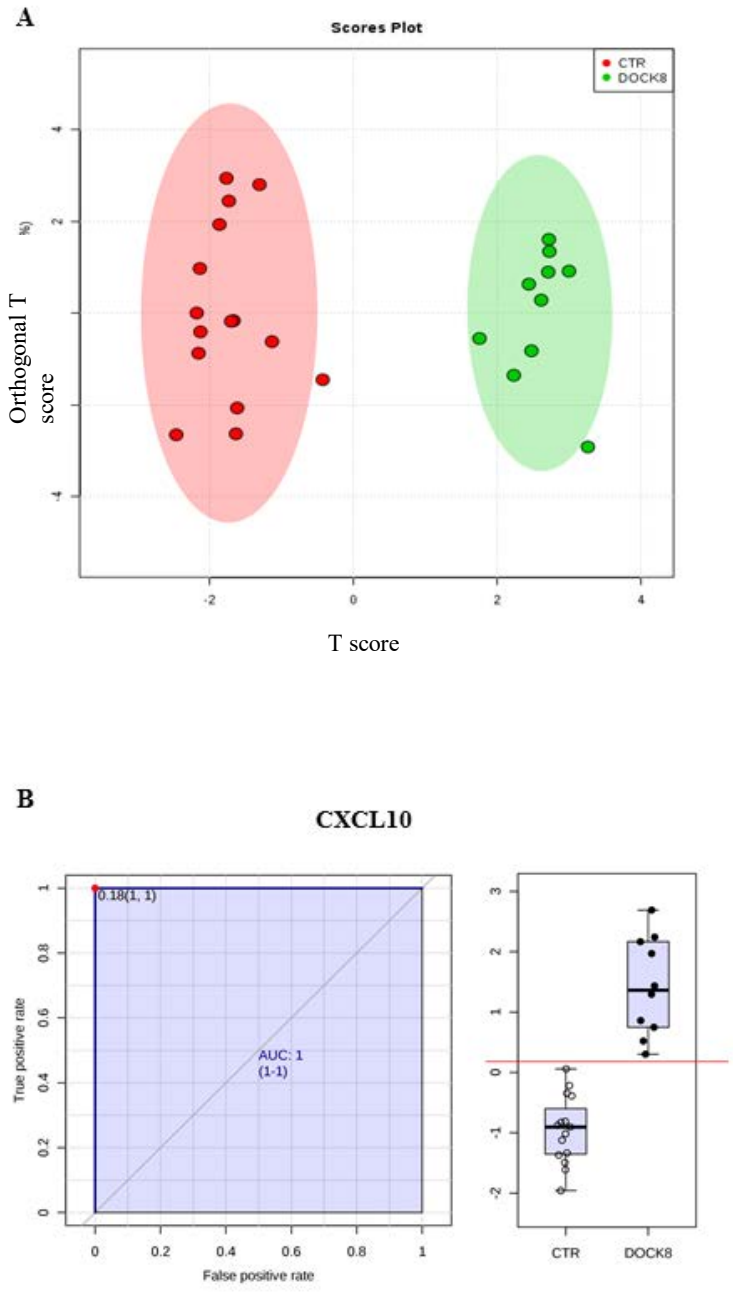

Figure 0.3: Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) for binary comparisons and area under the curve (AUC) in DOCK8 deficient patients.(A) Comparison between DOCK8 deficient patients and healthy controls with a calculated space Q2=0.864 that represents the level of differential expression of the panel analytes (B) CXCL10 is up-regulated with AUC: 1 in patients with DOCK8 deficiency

The separation between the two groups represents the degree of random relative expression in the whole panel due to DOCK8 deficiency (Q2 = 0.864) **(Error! Reference source not found.A**). A nalytes are ordered in Error! Reference source not found.**B** based on their contribution in separating the two groups. Among CXCL10, TNF-A, CSF3, CCL22, CX3CL1, IFNG, and TGFA that were above the volcano plot's cutoff values, CXCL10 was evaluated as a potential biomarker using the support vector machine (SVM) model ,to generate a receiver operating characteristic (ROC) curve, where the area under the curve (AUC) amounted to 1**(Error! R eference source not found.B**) . Another OPLA-DA based binary comparison between Ctrl and AD groups showed a Q2 of 0.32. (Error! Reference source not found.**C**) The relatively narrow spacing between the two groups indicates the lower ability of this profile to distinguish between the AD and ctrl individuals. As suggested by the volcano plot, the detected unique cytokine, EGF, which was downregulated in AD patients, had a ROC and AUC of 0.859 (Error! Reference source not found.**D**).

Since in this chapter, the strategy was to find disease-specific biomarker(s) able to differentiate between DOCK8 deficiency and AD, the binary comparison was performed which revealed clear cluster separation in OPLS-DA with a Q2 value of 0.693 **(**

Figure 0.4**A**). Comparison between all three groups also showed comparable separation but some overlapping was observed between the AD group and ctrl **(**

Figure 0.4**B)** may be due to mild eczema seen in the ctrl group. Interestingly, CXCL10 and TNF-A were the only signature cytokines, which were above the volcano plot's cutoff values and were significantly upregulated in DOCK8-deficient patients. The typified linear combination of these cytokines will be a potentially robust differentiating tool between DOCK8 and AD with AUC 0.978 **(**

Figure 0.4**C, D)**

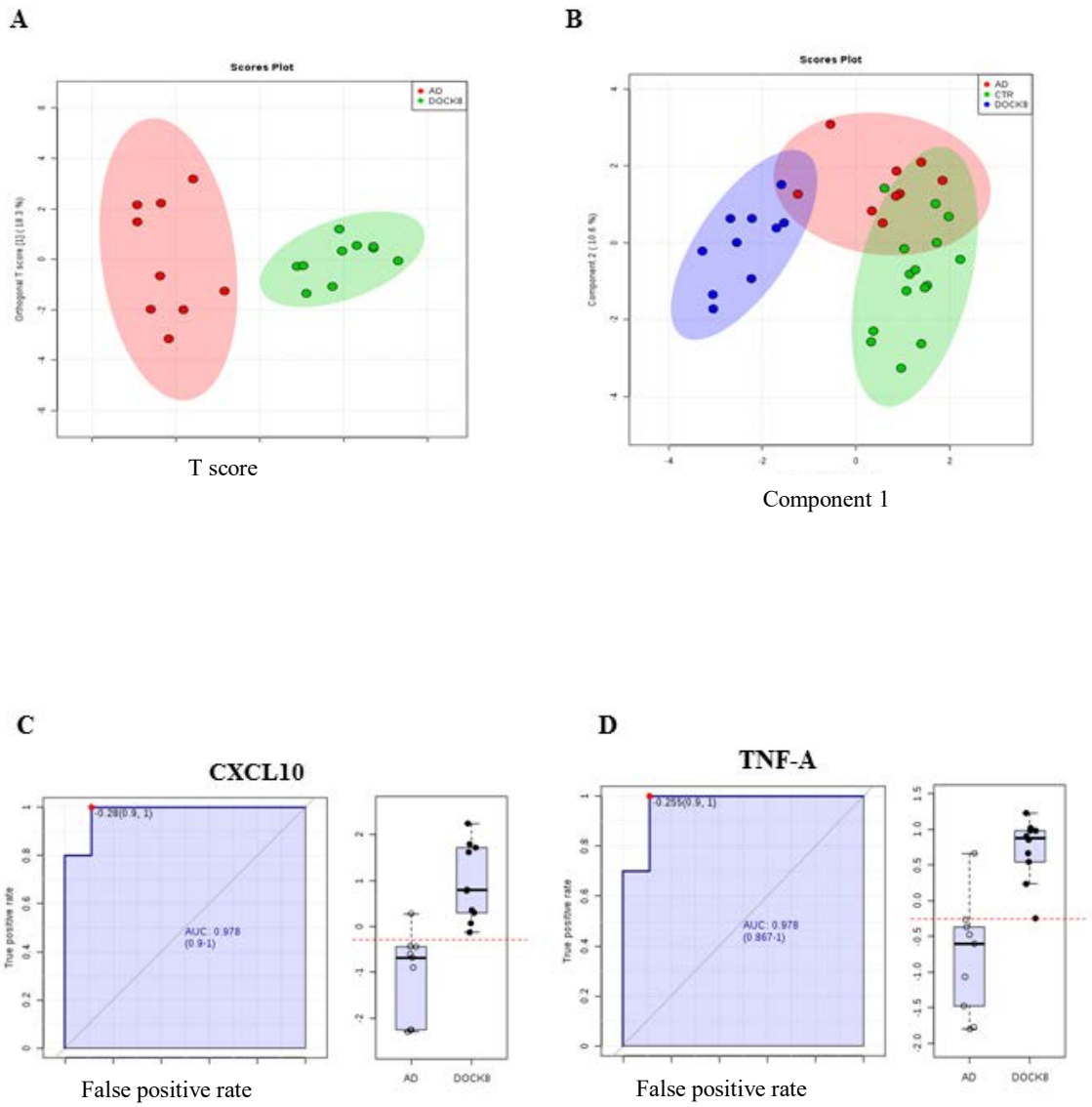

Figure 0.4: Orthogonal Projections to Latent Structures Discriminant Analysis (OPLS-DA) for binary comparison and area under the curve (AUC) in patients with DOCK8 deficiency, atopic dermatitis (A) Score plot comparison between AD patients and DOCK8 patients. (B) Overall comparison between AD patients, DOCK8-deficient patients and ctrl. (C) CXCL10 is up-regulated in DOCK8 deficient patients compared to AD patients, with AUC 0:978. (D) Tumor necrosis factor alpha (TNF-A) is up-regulated in DOCK8 patients compared to AD patients, AUC- 0.978.

Table 0.3: Differential significance in 39 cytokines

	DOCK8 (n=10)		AD (n=9)		Ctrl (n=15)		DOCK8_Ctrl	AD_Ctrl	DOCK8_AD
Cytokine/ Chemokine	Mean	SEM	Mean	SEM	Mean	SEM	p-value	p-value	p-value
EGF	119.81	28.36	91.53	28.36	244.52	35.50	0.14	**0.02**	0.67
FGF-2	52.35	13.25	114.48	44.90	117.31	27.74	0.31	1.00	0.43
CCL-11	152.58	23.96	97.61	19.15	162.67	22.01	1.00	0.13	0.19
TGF-A	9.18	3.79	9.16	3.04	6.48	1.74	0.06	0.23	0.81
GSF3	124.30	15.07	104.37	26.07	55.66	9.30	**0.01**	0.13	0.48
FLT3LG	32.01	18.97	22.32	19.28	20.14	8.57	0.88	0.47	0.68
CSF2	33.29	5.18	56.44	20.02	35.25	4.45	0.99	0.32	0.46
CX3CL1	57.60	12.94	207.51	55.06	251.22	53.88	**0.02**	0.81	0.14
IFNA	30.33	9.99	42.59	22.61	36.74	12.33	0.85	0.86	0.60
IFNG	16.35	5.09	62.98	27.87	53.77	14.62	0.20	0.87	0.13
CXCL2	7894.64	1249.41	5663.77	1652.69	5520.44	1482.61	0.57	1.00	0.68
IL-10	26.70	6.31	12.68	4.17	2.26	1.24	0.28	0.79	0.51
CCR3	23.15	11.95	142.41	63.59	75.96	43.04	0.72	0.84	0.46
IL12B	3.00	2.05	20.41	8.29	3.86	2.75	0.99	0.32	0.25
CCL22	2720.43	573.68	1838.89	223.77	1220.57	107.58	**0.01**	0.43	0.20
IL-12p70	6.64	4.82	42.04	28.78	47.11	20.37	ns	ns	ns
IL-13	14.15	6.64	135.06	76.43	62.41	39.07	0.64	0.39	0.15
IL-15	6.71	2.87	3.57	1.46	2.83	1.59	0.87	0.91	0.64
CD40LG	3205.41	1103.50	1354.48	360.54	4358.72	803.22	0.77	0.05	0.26
IL-17A	7.49	2.06	23.80	10.93	19.44	5.73	0.34	0.43	0.07
IL-1RA	93.87	42.57	213.46	103.00	109.94	67.94	0.52	0.96	0.35
IL-1A	290365.91	161683.29	801859.10	474855.45	2400302.99	2034028.62	0.58	0.74	0.97
IL-9	3.86	1.12	6.40	2.72	1.33	0.51	0.80	0.42	0.77
IL-1B	0.02	0.00	9.98	8.10	1.30	0.79	ns	ns	ns
IL-2	0.06	0.05	2.83	1.35	2.97	1.50	ns	ns	ns
IL-3	0.61	0.15	0.88	0.40	0.12	0.07	ns	ns	ns
IL-4	0.06	0.06	2.86	2.43	5.11	4.56	ns	ns	ns
IL-5	7.40	2.45	30.99	14.31	23.91	10.20	0.16	0.70	0.07
IL-6	28.78	12.96	31.04	24.42	5.92	2.60	0.73	0.69	0.99
IL-7	15.85	2.96	15.57	8.79	3.61	1.08	0.59	0.23	0.59
IL-8	24.20	2.39	29.27	8.70	26.00	6.14	1.00	0.93	0.91
CXCL10	1480.32	476.60	230.42	55.11	143.01	18.21	**0.00**	0.75	**0.00**
CCL-2	739.58	211.76	393.15	47.52	513.91	81.36	0.29	0.80	0.14
CCL-3	13.32	2.33	24.72	7.19	23.99	3.99	0.14	0.83	0.08
CCL-4	47.13	6.59	76.52	25.48	62.52	15.07	0.75	0.60	0.29
TNF-A	32.98	7.95	20.77	6.65	14.82	3.20	**0.03**	0.74	0.22
TNF-B	17.80	9.11	290.57	152.81	153.60	98.51	0.39	0.99	0.43
VEGF	319.24	59.76	339.93	122.33	338.44	70.57	0.97	> 0.99	0.98
IL-31	4363.38	1297.52	3780.75	1450.86	648.83	158.36	**0.02**	**0.01**	0.26

The statistical analysis was performed using one way ANOVA, post hoc Tukey's method,stastical s significance with p-value <0.05 and ns= not significant

2.5 Discussion

While significant progress has been made, the molecular mechanisms underlying specific cytokine functions remain largely unknown. The last decade has witnessed accumulating evidence supporting the central role of cytokines in immune surveillance as it relates to autoimmune diseases. It is essential to identify DOCK8 deficiencies before serious life-threatening complications arise. Diagnosis can be difficult in infants and young children because of coinciding laboratory findings and clinical profiles with AD and other types of HIES. There is global interest in identifying biomarkers that are disease-specific for the various clinical and molecular subtypes of HIES. The combination of recurrent viral infections, atopy, food allergies, consanguinity, and hyperactivity strongly suggests the diagnosis of DOCK8 deficiency. Supportive treatments including prophylactic antibiotics, nutritional support, and immunomodulatory agents remain unsatisfactory, and patients usually have very short life expectancy. Due to high morbidity and mortality associated with DOCK8 deficiency, curative treatment using hematopoietic stem cell transplantation is becoming the last preferred choice, resulting in successful immune reconstitution. (39) In this study, among the 39 cytokines, seven (CXCL10, TNF-A, GCSF, CX3CL1, MDC, EGF, and IL-31) were differentially expressed (

A-G) CXCL10 is a chemokine which is known to be highly expressed in various diseases,(40) pneumonia triggered by viral and bacterial infection (41) as well as in asthma.(42, 43) Upregulation of this cytokine in the DOCK8-deficient cohort seems to be associated with the commonly seen asthma, infections, allergies, and pneumonia phenotypes seen in these patients. **(**

A) In contrast, TNF-A is a multifunctional pro inflammatory cytokine, known to mediate tumor initiation, psoriasis. In contrast, TNF-A is a multifunctional pro inflammatory cytokine, known to mediate tumor initiation, psoriasis, AD and autoimmune diseases and participates in cell survival/death, and cancer.(44) Upregulation of TNF-A **(**

B) seems to be contributing towards the pruritus and immune dysregulation observed in DOCK8 patients. Furthermore, CSF3 is a glycoprotein linked with proliferation and maturation of neutrophils. (45) The role of CSF3 is evident in various cancers.(46, 47) Dehqanzada et al. in 2006 demonstrated that CSF3 was significantly overexpressed in breast cancer patients.(48) Higher expression of CSF3 **(**

C), demonstrated in those studies supports the role of immune responses, favoring tumor growth and progression which is in agreement with disease phenotypes seen in the DOCK8 cohort. Moreover, CX3CL1 is known to be associated with various inflammatory conditions like AD (49) and allergic asthma.(50) It was downregulated in DOCK8 patients when compared to ctrls **(**

D). CCL2 was shown to be involved in various immune responses and elimination of pathogens (51), allergic reactions (52)and inflammation in the lungs.(53) Hence upregulation of CCL2 (

E) is consistent with the clinical features (infections, allergies, and pneumonia) seen in the DOCK8-deficient patients.

Atopic dermatitis is a chronic inflammatory skin disease with frequent recurrences, and cytokines appear to play a significant role in its complex pathogenesis.(54, 55) EGF is

reported to regulate fundamental functions in mammalian cells including survival, migration, proliferation, and homeostasis. This cytokine is expressed at the site infection, and its primary function is the protection of the skin against bacterial colonization mediated through the EGFR signaling. (56-58) It has been shown that, EGF shows to be significantly down-regulated in AD patients, but not in DOCK8-deficient patients, when compared with ctrls **(**

F), hence, low levels of EGF may impair proliferation and anti-inflammatory immune response in the skin. IL-31 is a recently discovered cytokine produced by Th2 cells, and elevated levels of IL-31 are correlated with increased serum IgE levels and disease severity and are responsible for the induction of pruritus in the AD patients.(54) Recently, it has been shown that topical administration of EGF suppresses immune response and protects skin barrier in Dinitrochlorobenzene (DNCB) induced AD in NC/Nga mice This effect appeared to be the result of EGF up regulation of epidermal proteins and down regulation of thymic stromal lymphopoietin (TSLP) mediated mast cell and Th2 cell activation.(56-59) Therefore, we propose that in subsets of patients with EGF deficiency, replacement therapy with topical or systemically administered EGF may be of clinical value. CXCL10 and TNF-A were identified as potential biomarkers differentiating DOCK8 deficient and AD patients **(**

Figure 0.4**).** The reliability of these cytokines as diagnostic markers should be confirmed by questioning more massive datasets, to assess whether these cytokines are specific to DOCK8 gene expression or resulting from secondary disease complications.

A binary analysis was performed in DOCK8-deficient patients with and without one of the following clinical manifestations: asthma, bronchiectasis, molluscum contagiosum infection, sclerosing cholangitis, thrush candidiasis, warts, sinusitis, rhinitis, and malignancy. For instance, CSF3 was upregulated in DOCK8 patients with warts and molluscum contagiosum, while CX3CL1 and IFNG were downregulated in the DOCK8 patient with malignancy. Moreover, **Figure A2.3** represents volcano plots for binary comparisons between AD vs ctrl and DOCK8 vs AD, also demonstrates the potential biomarkers EGF, CXCL10, and TNF-A. As shown in Error! Reference source not found., neither of the potential biomarkers, CXCL10 a nd TNF, was over expressed in any of these binary analyses. Instead, they were downregulated in association with bronchiectasis and malignancy, suggesting that these biomarkers are very likely to be related mainly to the disease mechanism caused by DOCK8 deficiency.

These findings should be further studied in other HIES to determine their specificity.

CXCL10 is one of the CXCR3 ligands secreted by CD4+, CD8+ T cells, natural killer (NK), or NK-T cells in response to INFG. The average CD4+/CD8+ ratio in DOCK8 patients was higher (2.8 ± 0.99) than AD groups (1.43 ± 0.14) The C-terminal of CXCL10 has a high affinity for cell surface heparan sulfate glycosaminoglycan (HS-GAG), which is expressed on epithelial, endothelial, and hematopoietic cells. The higher circulated level of CXCL10 in blood is due to either a higher competitive binding with a viral protein inflammation, (60) as it attracts the activated T lymphocytes, which are the only inflammatory cells expressing the chemokine receptor CXCR3), or DOCK8 inhibition of HS-GAG synthesis. From the clinical data collected for this study **(Table 0.2)**, the spectrum of infections observed in DOCK8-deficient patients such as pneumonia (100%), staphylococcal infections (100%), and bronchiectasis (50%) suggests a connection between pathogens binding to HS-GAG, and the degree of inflammation, which increases the circulated CXCL10.(61) CXCL10 levels in AD

patients were not as high as in DOCK8 patients, probably because AD patients lack this severe and complex spectrum of infections usually seen in DOCK8-deficient patients. However, Brunner et.al (2017) showed in his studies that CXCL10 levels were up-regulated in AD patients, which could be the contributing factor for the atopic diseases seen in both cohorts, as they share many symptoms, especially eczema.(62)

Th2 cells producing IL-4, IL-5 and IL-13 are involved in humoral immunity against parasites and in allergic reactions. (63, 64). IL-5 plays a crucial role r in innate and acquired immune response and eosinophilia. (65) Although IL-4 and IL-13 are similar cytokines, they have some differences for example, IL-13 does not cause proliferation of T cells or differentiation of naïve T cells towards Th2 cells as IL-4 does, but the activation of mast cells by IL-13, is weaker, compared to IL-4.(66) Th2 cells are known to play an important role in the pathogenesis of allergic asthma, (67) IL-4, IL-5 and IL-13 produced by allergen specific Th2 cells probably contribute in our study population to immune response directed against specific environmental antigens. This could be the reason that these cytokines were not statistically significant in both our cohorts.

Th17 cells are essential for host defence against extracellular pathogens distinct from those targeted for Th1 and Th2 cells including pathophysiology of immune inflammatory disorders.(68) Few studies have been reported connecting severity in inflammation and Th17 cell mediated immune responses.(68, 69) Th17 cells have shown to exhibit both pro and anti-tumor functions. These cells have been shown to grow tumors by inducing immune effector cell recruitment within the tumor and they also produce IFNg in the tumor that will result in inhibition of tumor growth.(70) IFNg is also critical for innate and adaptive immunity against viral, intracellular bacterial infections and tumor control.(71) In this study, IFNg was down-regulated in the DOCK8 cohort when compared to AD, suggesting poor immunity in these patients.

In summary, significant differential expression of cytokines CXCL10 compared to both AD and ctrl cohorts and CSF3, CCL22, CX3CL1, and TNF-A compared to the ctrls were identified in DOCK8 deficiency, which possibly contributes to increased susceptibility to infection and cancer. CXCL10 and TNF-A seem to be important biomarkers in differentiating between DOCK8-deficient and AD patients, whereas EGF was found to be a useful biomarker in AD patients. Cytokine IL-31 expression was comparable in both cohorts thus contributing toward pruritus, which is common to both groups. The unique expression profiles including CXCL10, CSF3, CCL22, CX3CL1, and TNF-A that were detected in patients with DOCK8 deficiency probably reflect disease-specific perturbations in multiple cellular processes and pathways leading to a predisposition to infections, allergy, and malignancy seen in these patients. Hence, the linear combination of three "CXCL10, TNF-A, and EGF" typified biomarkers signature could lead to a unique scoring system for early effective diagnosis and distinction of DOCK8 deficiency vs atopic dermatitis.

More broader cytokine profiling may reveal additional biomarkers linking these disorders with their clinical phenotypes thus facilitating improved understanding of disease pathogenesis. Scientific and clinical approach together will be required to gain novel insight into fundamental immunological mechanism in DOCK8 deficiencies. Complex phenotype and a broad range of combined immunological abnormalities are a major challenge in providing the proper optimal treatment to these patients. Since DOCK8 and AD patients have overlapping symptoms, their treatment management is also similar. These patients were on curative treatments like use of prophylactic antibiotics, antihistamines and topical steroids. Since samples were collected while they were clinically stable, there is no expected effect on the metabolome or the results. In summation, such imbalanced events may have a major impact on the development and progression of the disease. Further studies on the mechanism by which these cytokines promote the genesis of this disorder may yet further apply to the

ongoing molecular cellular and network reorganization that follow other types of immune deficiencies. Apart from the cytokine biomarkers, metabolomics biomarkers, were studied and discussed in chapter 4. Hypotaurine, 3-hydroxyanthranillic acid and glycyl-phenyalanine were identified as potential biomarkers specific for DOCK8 deficiency; perturbations in tryptophan degradation and increased availability of aspartate suggest a link of DOCK8 deficiency to oncogenesis. Additionally, perturbations in taurine and dipeptides metabolism suggest altered antixidation and cell signaling states in DOCK8 deficiency.In the next chapter, a comprehensive metabolomics method was built for metabolomics biomarker study in DOCK8 deficiency vs atopic dermatitis.

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2.7 Summary of Chapter 2

- The aim of this study was to identify cytokine biomarkers in DOCK8-deficient and AD patients
- Significant differential expression of cytokines CXCL10, CSF3, CCL22, CX3CL1, and TNF-A were identified in DOCK8 deficiency, which possibly contributes to increased susceptibility to infection and cancer
- CXCL10 and TNF-A seem to be important biomarkers in differentiating between DOCK8‐deficient and AD patients
- EGF was established to be a good biomarker in AD patients.
- Cytokine IL-31 expression was comparable in both cohorts thus contributing toward pruritus, which is common to both groups
- The linear combination of three typified signature biomarkers, "CXCL10, TNF-A, and EGF" could lead to a unique scoring system for early effective diagnosis and distinction of DOCK8 deficiency vs atopic dermatitis

CHAPTER 3: A TARGETED METABOLOMICS APPROACH FOR CLINICAL DIAGNOSIS OF INBORN ERRORS OF METABOLISM

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3.1 Introduction

Metabolomics signifies the comprehensive assessment of endogenous and exogenous small molecules, (metabolites) and systematically identify and quantify them in various biological systems under consideration.(1, 2) Since the metabolome is responsive to changes induced by a diverse array of genetic and non-genetic factors including nutrition and therapeutics, it is an ideal approach to track, monitor and understand the connection of these influences to phenotype. (3, 4) The sensitivity of metabolic profile is the primary challenge that hampers the quality of data and biological interpretation.(5) Integrating the data set of metabolomics with genomics, transcriptomics, and proteomics, adds a more reliable and informative dimension to the phenotype, leading to a better understanding of the disease pathophysiology. (3, 4) The association between the metabolic changes, patient phenotype, pathological status and treatment, is well established and has enhanced the application of metabolomics in various medical fields. (6-9) Since every molecule has a metabolic fate, there is a panel of metabolites that represents the end products of known biochemical pathways. Thus, any biological disturbance should potentially have a distinct metabolic imprint indicative of the current state, reflecting the net effects of endogenous and exogenous molecules on anabolism and catabolism.(10)

The chemo-physical diversity of metabolites makes global metabolomics profiling an analytical challenge. Sample pretreatment is crucial in metabolomic studies, as metabolism is dynamic by nature metabolomic profiling is influenced by sample type, gender, diet, lifestyle, sample collection conditions, storage conditions, metabolism quenching and extraction because any contamination or degradation will adversely affect degradation will adversely affect the analytical results.(11) Hence these factors should be controlled, and an appropriate number of samples should be used in pilot studies.(12) In Gas chromatography-mass spectrometry (GC-MS), the degradation of thermo labile compounds can have a significant

impact on the data quality; hence it is advisable to use liquid-based techniques for such analytes. Matrix effect can be solved with the utilization of an internal standard and by proper evaluation of matrix effect during method development .(12) Several analytical platforms are used in metabolomics, but none fulfills the criteria of being an ‘ideal global metabolic profiling technique' as biological samples include thousands of metabolites that vary, not only in concentration but also in their physical and chemical properties .(1, 13).

Recently, various metabolomics methods have been introduced for analyte detection and clinical biomarker discovery. Miyamoto et al. (2015), used GC-TOF-MS based metabolomic profiling to evaluate the prognosis in patients with lung cancer and showed up regulation of certain sugars and amino acids in affected individuals when compared with ctrls.(14) Also, analysis of a wide range of metabolites, ranging from high to low molecular weight and from hydrophilic to hydrophobic was demonstrated using LC-MS/MS. (15, 16) A nuclear magnetic resonance (NMR) based combined breathomics and metabolomics study revealed a marked difference between healthy and asthmatic children.(17)

Recent advances in instrument development in NMR, MS, and Ultra Performance Liquid Chromatography (UPLC) combined with bioinformatics has paved the way for sophisticated and more robust metabolomics studies over the last decade.(18, 19) Diagnostic metabolic profiles to identify candidate biomarkers in animal and human tissue was made possible by combining the separation analytical technology of UPLC with MS.(20) Liquid chromatography-mass Spectrometry (LC-MS/MS) has been widely used for global metabolic profiling, as indicated in some published studies.(21, 22)

Several hundred inborn errors of metabolism (IEM), which are primary genetic disorders and involve intermediary metabolism of small and large molecules have been characterized clinically and biochemically. Most IEMs have a severe impact on affected individuals leading to varying degrees of developmental and physical retardation, organ function derangement

and even death, as especially seen in severe mitochondrial and urea cycle disorders.The implementation of newborn screening for an increasing number of IEM showed birth prevalence as high as 1 in 500 live births in some populations. Enzyme deficiencies, the hallmark of IEMs, usually lead to the accumulation of toxic (precursor) compounds and depletion of certain product compounds, thereby affecting proximal and distal cellular processes and resulting in a unique combined clinical and biochemical disease phenotype. The unique metabolic profiling can be useful in clinical care, biomarker discovery for several common and rare human diseases, as well as in understanding disease mechanisms and response to therapy. Currently, clinical metabolic profiling of acylcarnitines, amino acids, very long chain fatty acids, steroids and organic acids are routinely performed in newborn screening and biochemical genetics laboratories worldwide.(23, 24) These assays are targeted to a group of disorders and are performed individually using different platforms. Few laboratories have already developed and adopted limited clinical metabolomics assays useful in the evaluation of patients suspected of having metabolic diseases. (7, 9, 25) However, conventional IEM diagnosis procedures are based on a series of sequential and segmented biochemical assays on various analytical platforms. This diagnostic strategy is slow, time consuming and complex, whereas optimal patient management requires an improved speed of biochemical tests to allow early diagnosis for better monitoring and management of IEMs. Comprehensive and quantitative metabolomics is increasingly assuming a more significant role in exploring novel ways to diagnose, treat and prevent nutrition-related diseases such as obesity, diabetes mellitus, and some IEMs. (26, 27) like phenylketonuria(PKU),maple syrup urine disease(MSUD),very long chain acid dehydrogenase deficiency(VLCADD), glutaric type-1(GA-1), glutaric type-2(GA-2), tyrosinemia type-1(TYR-1) and tyrosinemia type 2(TYR-2).

For example, insulin resistance (IR) is a known significant early risk marker for metabolic syndrome and cardiovascular disease and can precede dysglycemia in prediabetics and patients with type 2 diabetes mellitus (T2DM) by some years. In contrast to the traditional glycated hemoglobin (HbA1c), new non-glycemic {a-hydroxybutyrate, linoleoyl-glycerophosphocholine (L-GPC), oleic acid} biomarkers, in combination with insulin, provide a novel comprehensive and more robust measure of insulin resistance in a single assay (Quantose IR™). These biomarkers (alpha hydroxybutyrate, L-GPC, and oleic acid) correlate with early beta cell dysfunction, impaired glucose tolerance and increasing lipolysis respectively. In addition, increased insulin level is characteristic of IR and is an independent risk factor for type 2 diabetes and cardiovascular disease.(28, 29)Recently, additional insight into the pathogenesis of phenylketonuria was gained using GC-MS and NMR based metabolomics. Analysis of urine samples from these patients demonstrated multiple perturbations in arginine, proline, alanine, aspartate, and glutamate metabolism pathways. (9) This chapter details the development of an LC-MS/MS-based comprehensive targeted metabolomics panel which encompasses a broad range of clinical and biochemical genetic disorders (such as disorders of metabolism of amino acids, organic acids, bile acids, polyamines, neurotransmitters, sugars, and mitochondrial fatty acid oxidation). The method is applicable for different biological matrices such as whole blood, serum, dried blood spots (DBS) and tissues. The sensitivity, selectivity, and linearity of this LC-MS/MS method were evaluated, including the stability of the measured metabolites under defined preparation and analysis conditions. (15, 30, 31) Since DBS represent the maximum metabolic level of analytes that remain stable and are quantifiable, (32, 33) the method was validated on DBS from eight different groups of patients with a known diagnosis of IEM.

3.2 Aims

The aims of this study were as follows:

1) To develop and standardize a metabolomics method, utilizing LC-MS/MS for metabolomics profiling in DOCK8 deficient and Atopic dermatitis patients.
2) Validate this method, as per the United States Food and Drug Administration (US FDA) regulations.
3) Test the applicability of this method on three different matrices: DBS, serum and tissues.
4) Validate the developed method on eight known diagnosis of IEM.
5) Validate the method on DBS with eight known IEM.
6) Build a reference range for DBS.

3.3 Materials and Methods

3.3.1 Chemicals and reagents

Metabolite standards and reagents were obtained from Sigma Chemicals (St. Louis, MO) at a minimum purity of 98%). All organic solvents and water used in sample and mobile phase preparations were LC-MS/MS grade and obtained from Fisher Scientific (Fair Lawn, NJ).Isotope labeled internal standards were purchased as follows:

Isotope labeled standards	
Pterin	Schricks Laboratories (Postface, Switzerland).
L-Monapterin	Schricks Laboratories (Postface, Switzerland).
2-Amino 1,6-Hexandioc-D3	Cambridge Isotope, Inc (Woburn, MA).
Guanosine-15N5	Cambridge Isotope, Inc (Woburn, MA).
Inosine-15N4	Cambridge Isotope, Inc (Woburn, MA).
D-Fructose (2e13C)	Cambridge Isotope, Inc (Woburn, MA).
Citric acid-D4	Cambridge Isotope, Inc (Woburn, MA).
L-Citrulline-D7	Cambridge Isotope, Inc (Woburn, MA).
Adenosine-C13	Cambridge Isotope, Inc (Woburn, MA).
Methylmalonic acid-D3	Cambridge Isotope, Inc (Woburn, MA).
Alanine-D1	Cambridge Isotope, Inc (Woburn, MA).
2- Deoxyadenosine-C13	Cambridge Isotope, Inc (Woburn, MA).
GlucoseeD7	Cambridge Isotope, Inc (Woburn, MA).
Alanine-D4	ChromSystems (Grafelfing, Germany
Arginine- D7	ChromSystems (Grafelfing, Germany
Aspartic acid-D3	ChromSystems (Grafelfing, Germany
Citrulline-D2	ChromSystems (Grafelfing, Germany
Glutamic acid-D5	ChromSystems (Grafelfing, Germany
Leucine-D3	ChromSystems (Grafelfing, Germany
Methionie-D3	ChromSystems (Grafelfing, Germany
Ornithine D6	ChromSystems (Grafelfing, Germany
Phenylalanine-D5	ChromSystems (Grafelfing, Germany
Tyrosine- D4	ChromSystems (Grafelfing, Germany
Valine-D8	ChromSystems (Grafelfing, Germany
C0-Carnitine-D9	ChromSystems (Grafelfing, Germany
C2-Carnitine-D3	ChromSystems (Grafelfing, Germany
C3-Carnitine-D3	ChromSystems (Grafelfing, Germany
C4-Carnitine-D3	ChromSystems (Grafelfing, Germany
C5-Carnitine D9	ChromSystems (Grafelfing, Germany
C6-Carnitine-D3	ChromSystems (Grafelfing, Germany

C8-Carnitine- D3	ChromSystems (Grafelfing, Germany
C10-Carnitine-D3	ChromSystems (Grafelfing, Germany
C12-Carnitine-D3	ChromSystems (Grafelfing, Germany
C14-Carnitine-D3	ChromSystems (Grafelfing, Germany
C16- Carnitine-D3	ChromSystems (Grafelfing, Germany
C18-Carnitine-D3	ChromSystems (Grafelfing, Germany

3.3.2 LC-MS/MS analysis

In this LC-MS/MS method, an Acquity UPLC-XEVO TQD tandem mass spectrometer (Waters Corporation, USA) was used, where analytes were separated by reversed phase chromatography using Acquity UPLC C18, 1.7 mm, 2.1 mm x 100 mm column (at ambient temperature). Each sample was analyzed twice; in positive and negative ionization modes. In positive mode analysis, the mobile phase consisted of (A) 0.1% acetic acid and (B) 50% acetonitrile (ACN) and 50% Methanol (MeOH). The mobile phase, for the positive mode, was ramping from 2% to 95% for 10 min, held for 1 min at 95% then mobile phase A was ramped back to 2%, to regenerate the column for the next run. In negative mode, the mobile phase was composed of (A) 0.1% tributylamine (TBA), 0.03% acetic acid, 10% MeOH and (B)100% ACN. Subsequently, mobile phase for negative mode was ramping from 5% to 70% for 13 min, held for 1 min at 70% and then the mobile phase A, ramped back to 5%, to regenerate the column for the next run. The samples were run in the positive mode, first and then run on the negative mode, with an intermediate automated washing step to avoid any sample carryover. The total run time for each sample in each mode was 15 min at a flow rate of 0.3 mL/min. The samples were stored in the auto sampler at 4ºC, and the injection volume was 10 ml.

The targeted compounds were prepared in 50% methanol (400 mM) and infused into XEVO TQD (Waters Corporation, USA) for optimization. The source and desolvation temperatures were set at 150 ºC and 250º C, respectively, while the desolvation gas was set at 500 L/h to tune molecules in both (positive and negative) polarity modes. The specific tuning

parameters, such as ionization polarity, precursor and product ions, cone voltage and collision energy (CE) were obtained for each analyte. The eluted metabolites were analyzed under the optimal MS conditions listed in **Table A1.2** using an electrospray ionizing. The cone voltage ranged from 18 to 170 V, and the collision energy ranged from 7 to 65 eV. Common MS parameters were the same as the tuning conditions described above except the desolvation temperature and gas flow at 500 ^{0} C and 1000 L/h, respectively. A chromatographic method was developed to accommodate the best baseline separation for the targeted metabolites within 15 min of retention time, the gradient started at injection (zero dead volume). A mixture of all of these compounds was used to prepare a wide range of calibration curve (1e1000 nM) and a set of analytical quality control (QC) samples (25, 250,750 nM). The MS was maintained using a calibration kit and protocol as recommended by the manufacturer (Waters Corporation, USA).

3.3.3 Assay validation

Validation of the analytical method was performed according to the United States Food and Drug Administration (US FDA), and International Conference on Harmonization (ICH) guidelines.(15, 34, 35)

3.3.3.1 Specificity and sensitivity

For the metabolites listed in **Table A1.2**, calibration curves were prepared daily in serial dilution and spiked with standard internal mixture (IS). In each detection mode, a set of IS compounds were used to correct for the sample preparation and LC-MS/MS fluctuations as detailed in **Table A1.3**. The calibration curve linearity was evaluated over three consecutive days. A blank with internal standard (IS) mixturẽ, a standard metabolites mixture, and 7-10 points calibration curves were analyzed using the developed method. The calibration curves were generated by plotting the peak area ratio (area analyte /area IS) of the metabolite versus the nominal concentration of each analyte. The lower limit of quantification (LLOQ) was

defined as the lowest point in the calibration curve, where the signal was at least ten times greater than the standard deviation of the blank. The LLOQ accuracy should be within 80 to 120%, and day-to-day variation less than or equal to 20%. During optimization, target analyte fragmentation was minimized and the fragmentation of the isobaric matrix components was maximized, hence the precursor ion of the analyte passed through the collision cell with a minimum intensity loss, while the isobaric matrix components were eliminated by fragmentation in the collision cell. The minimal difference between retention times in matrix-free (solvent) and (serum) spiked samples (<0.1%) allowed confident, highly specific, peak identification. Also in the chromatography method, the first minute was excluded for eluting compounds to overcome the possible ion-suppression. Finally, to avoid any other potential source of signal fluctuation, only significant fold change at a cut off 2 with false discovery rate (FDR) corrected p-value <0.05 was considered.

3.3.3.2 Intra and inter-day precision

The intraday variability was evaluated by preparing three independent fresh replicates (n ¼ 6) of each QC sample, a mixture of standard metabolites and internal standards prepared in the lab to yield concentrations of 25, 250, 750 nM. Over three different days, the inter-day validation was accomplished similarly. The accuracy was calculated as (mean measured concentration/nominal concentration) x 100%, and the variability was represented as the percent relative standard deviation (percentage RSD).

3.3.3.3 Stability

To study the metabolite solution stability, under different sample preparation and analysis conditions, a set of three QC samples (25, 250, 750 nM) were prepared and treated under the following conditions: room temperature (RT), at bench top for 6 h, 7 days in the auto sampler or refrigerator (4°C), 30 days storage in the freezer (-20°C) and 30 days storage in liquid

nitrogen (recognized as fresh). The long-term stability of these metabolites in DBS was evaluated by running a batch of 20 DBS of controls, at 0 times (fresh), 2 months and 4 months. The molecular stability was calculated as (area ratio of examined sample/area ratio of the fresh sample) x 100%.

3.3.3.4 Recovery

The recovery of the metabolites was studied through extraction, where plasma samples were spiked with a mixture of labeled internal standard extracted and then analyzed on this analytical platform. The recovery percentage was calculated as (area ratio of extracted metabolites from plasma/area ratio of metabolites in solution) x100%.

3.3.4 Biological sample collection and metabolite extraction.

3.3.4.1 Animal tissue samples

Heart, liver, kidney, muscle, and brain tissue samples were collected from five healthy male Sprague Dawley (SD) rats by following the protocol approved by the animal care and use committee (ACUC) at King Faisal Specialist Hospital and Research Center (KFSH&RC) (Study number: 2150,016). Upon sacrifice, the rat tissue samples were excised and washed quickly with cold phosphate buffered saline (PBS), snap frozen in liquid nitrogen, and then stored at 80^0 C. All tissues were processed together at the same time as detailed in a previous study, where ~100 mg of tissue was pulverized in a cell crusher under liquid nitrogen for metabolic extraction.(36) The polar metabolites were extracted by adding 1.0 ml of IS (250 nM) mixed with the extraction solvent (40% ACN, 40% MeOH and 20% water). The mixture was shaken at 1000 round per minute (RPM) for 1 h at 4 ° C in a thermomixer (Eppendorf, Germany). Following extraction, samples were spun down at 14,000 RPM on an Eppendorf centrifuge (Hamburg, Germany) for 10 min at 4°C, and the supernatant was transferred to fresh tubes to be evaporated in Savant™ SpeedVac Concentrator (Thermo scientific, USA).

This step was performed twice to enhance the extraction recovery. Then after the extracted solvent was evaporated to dryness in the concentrator, the dry extract was reconstituted in a 100 ml mixture consisting of the mobile phase (95% of 0.1% acetic acid and 5% of mixture of 50% ACN and MeOH) and stored at 4ºC for LC-MS/MS analysis.

3.3.4.2 Biological matrix: whole blood and serum

Whole blood samples were collected from 20 healthy adult individuals: 100 ml was aliquoted into Eppendorf tubes, 100 ml was spotted on Guthrie cards (Perkin Elmer) and the rest of the blood sample was centrifuged to obtain 100 ml serum. The polar metabolites were extracted from blood and serum with a mixture of IS (250 nM) and extraction solvent (50% ACN, 50% MeOH) as per the developed extraction protocol in section 3.2.4.1

3.3.4.3 Dried blood spots (DBS)

Initially, DBS samples from the same 20 healthy adult volunteers (Section 3.2.4.2) were punched (5 discs, 3.1 mm each) into a 96-well plate, and then metabolites were extracted using a mixtureof IS (250 nM) and extraction solvent (40% ACN, 40% MeOH and 20% water). The extraction procedure was completed as detailed in (section 3.2.4.1).The potential clinical usefulness of this method was further evaluated by running additional 200 random DBS samples obtained from the Newborn Screening & Biochemical Genetics Laboratory (NSBGL) at the KFSHRC, as per the ethics committee approval (Project# 2160027). As a part of the routine newborn screening panel, in Saudi Arabia, all of these 200 DBS samples were previously reported to be negative for 17 inborn errors of metabolism.(Phenylketonuria, Maple syrup urine disease, Arginosuccinase deficiency, Citrullinemia, 3-hydroxy-3-methylglutaryl-CoA lyase deficiency, Isovaleric acidemia, Methymalonic acidemia, Propionic acidemia, Beta ketothiolase deficiency, Glutaric type-1, Medium chain acyl CoA dehydrogenase deficiency, Glutaric type-3, 3-MethylcrotonylCoA carboxylase deficiency,

Galactosemia, Congenital hypothyroidism, congenital adernal hyperplasia, Biotindase deficiency)

In addition, 56 abnormal DBS samples from patients with 8 IEM disorders were collected; [phenylketonuria(PKU),maple syrup urine disease(MSUD),very long chain acid dehydrogenase deficiency(VLCADD), glutaric type-1(GA-1), glutaric type-2(GA-2), tyrosinemia type-1(TYR-1) and tyrosinemia type 2(TYR-2)]. In addition, 56 abnormal DBS samples from patients with eight different inborn errors of metabolism were collected. These samples failed the routine MS/MS-based newborn screening pipeline and were then confirmed as having one of the 8 disorders listed in **Table 0.1.** These samples were evaluated using this method to study the potential capability of this panel to detect and profile various IEMs.

3.3.5 Data and statistical analysis

3.3.5.1 Data acquisition, processing, and visualization

The sample delivery to the mass spectrometer was managed through the LC-MS/MS software of MassLynx, version 4.1 (Waters Corporation, Massachusetts, USA), which was also used for tuning and data acquisition. The peak integration and data analysis were performed using TargetLynx (Waters Corporation, Massachusetts, U S A). The area under the peak was used as the analytical signal for quantitative measurement of assay performance regarding linearity, sensitivity, and reproducibility. The tuning parameters and the retention times for the compounds are represented in **Table A1.2.**

3.3.5.2 Stastical Analysis.

The raw data was analyzed using MetaboAnalyst software version 3.0 (McGill University, Montreal, Canada). Features with more than 50% missing values were removed, while others

with missing values were replaced with small values (half of the minimum positive values in the original data) assumed to be above the detection limit. The data was then normalized to the equivalent internal standard's area under the peak, and then to the sample total sum to ensure normal distribution. To adjust for the differences among the study samples, data log transformation, and Pareto scaling approaches were used to make individual features more comparable. As the vast majority of the study analytes were Gaussian distributed, an unpaired two-tailed Student's t-test was used to compare the differences between two study groups (treated, non-treated), where the significance levels for metabolomics data were considered at a FDR corrected p-value < 0.05, and values were presented as mean ± SEM. The Sample Size Calculator for designing clinical research (http://www.sample-size.net) was used for the Mean-effect size analysis which was performed along all statistical analyses. The chemometric analysis was carried out using principal component analysis (PCA) and orthogonal partial least-squares projection to latent structure-discriminant analysis (orthoPLS-DA) (33).Orthogonal projection of Latent structures-discrimination analysis (OPLS-DA) is a supervised multiple regression analysis for identifying the discrimination between different datasets. The bar-graphs were generated using Graph Pad Prism V. 6 (34, 35). The FDR-corrected p values are represented on the figures as 0.0001 (***), 0.001 (**), and 0.05 (*). The statistically significant features between the study groups were used for pathway analysis and molecular mapping. Metabolic enrichment and pathway analyses were based on MetaboAnalyst (http://www.metaboanalyst.ca). The Rattus norvegicus pathway library was used. Cytoscape 3.4.0 on MetScape (http://www.cytoscape.org) was used for large-scale network analysis and the visualization of the integrated metabolism pathways

3.4 Results and discussion.

3.4.1 Method development and MS optimization

A library of 225 targeted and clinically relevant metabolites was obtained from commercial sources and used to optimize the LC- MS/MS instrument in multiple reactions monitoring (MRM) mode as shown in **Error! Reference source not found..**The compound specific c hromatographic and mass spectrometric parameters such as RT, precursor ion, product ion, collision energy (CE) and cone voltage are summarized in **Table A1.2.** The chromatographic parameters were developed in gradient reversed phase to obtain proper analytical peaks. The reproducibility of liquid chromatography is very important to distinguish between the molecules produced inside the ion source such as ATP dissociation to ADP and AMP causing endogenous artifacts. Representative over layed chromatograms in both positive and negative ionization modes are shown in **Error! Reference source not found.A and B,** respectively. The s tock solution of the standard mix was prepared at a concentration of 10,000 nM. Standard solutions (1, 5, 10, 25, 50, 100, 250, 500, 750, 1000 nM) were prepared by serial dilution of the stock solution with mobile phase mix. (95% of 0.1% acetic acid and 5% of mixture of 50% ACN and MeOH)

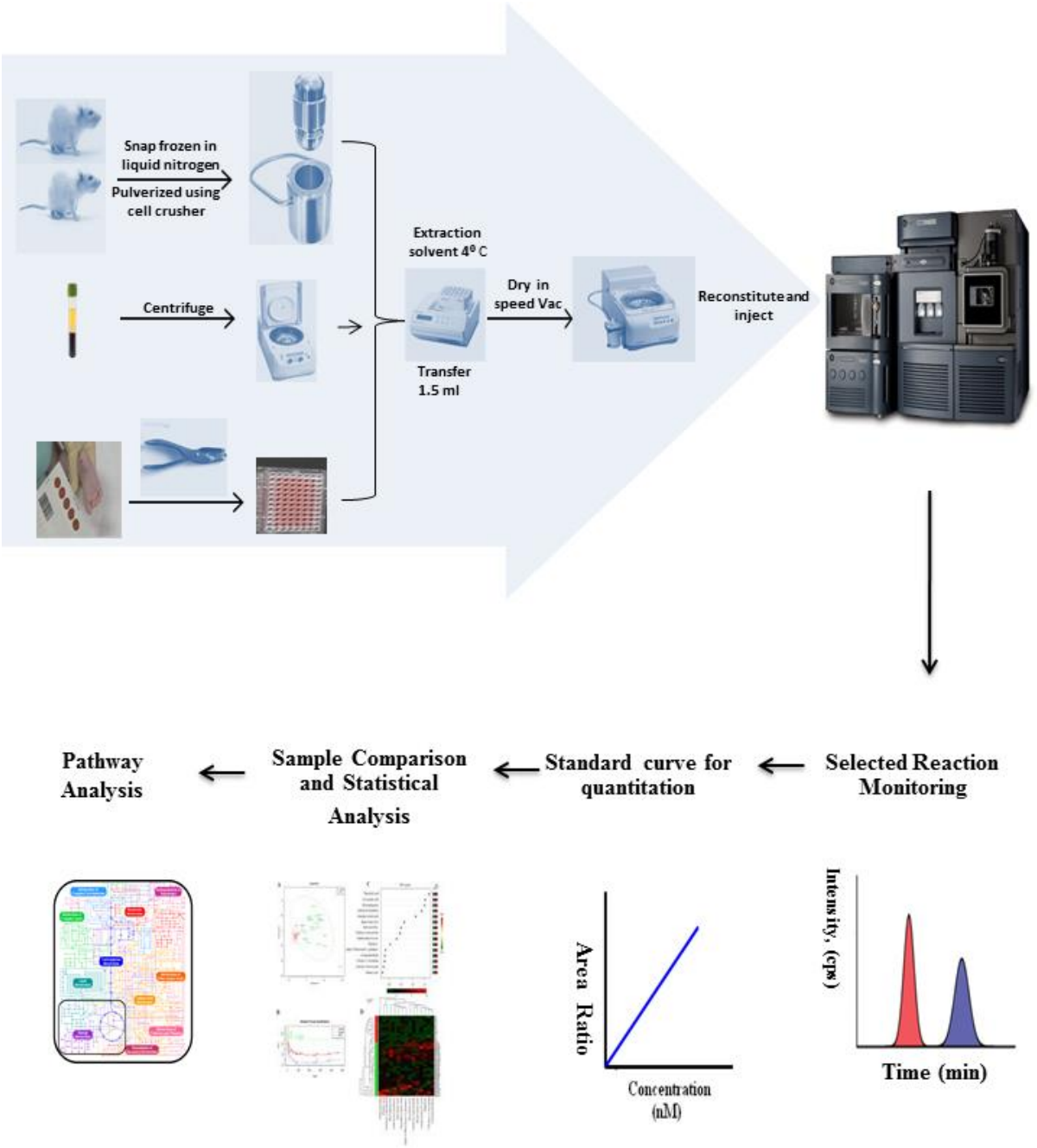

Figure 0.1: Workflow for targeted LC-MS/MS analysis of metabolites extracted from Dried Blood Spot (DBS), plasma, whole blood, and tissues. Tissue samples were frozen on dry ice, stored at -80ºC, and crushed in a crucible on liquid nitrogen just prior to extraction

Table 0.1: Diagnostic key markers in the 8 IEM panel (PKU, MSUD, VLCADD, MMA/PA, GA-1/2, TYR1/2)

Disease	Number of patients	Biochemical markers		Sex		Age (years±SD)
		Primary	Secondary	Male	Female	
PKU	15	Phe	Phe/Tyr	7	8	13.9±8.7
MSUD	10	Leu, Ileu, Val	Leu/Ala	6	4	6.7±5.2
VLCADD	10	Tetradecenoylcarnitine (C14:1)	C14/C16	5	5	3.0±1.58
MMA/PA	8	Propionylcarnitine (C3)	C3/C2, (urine methyl malonic acid, 3-hydroxy propionic acid, methylcitrate)	6	2	4.1±3.5
GA-1	2	Glutarlycarnitine (C5DC)	(urine glutaric & 3-hydroxyglutaric acids)	1	1	5±2.82
GA-2/ MADD	1	C4, C5			1	22±0
TYR-1	7	Tyr	Succinylacetone	6	1	15.1±4.87
TYR-2	3	Tyr	Urine 4-hydroxyphenyl lactate, 4-hydroxy phenylpyruvate	2	1	21±12.7

Abbreviations: PKU phenylketonuria , MSUD maple syrup urine disease, VLCADD very long chain acyl-CoA dehydrogenase deficiency, MMA/PA methylmalonic acidemia , PA propionic academia ,GA-1 glutaric acidemia type1, GA-2 glutaric acidemia type2, MADD multiple acyl-CoA dehydrogenase deficiency, TYR-1 tyrosinemia type1, TYR-2 tyrosinemia type2.

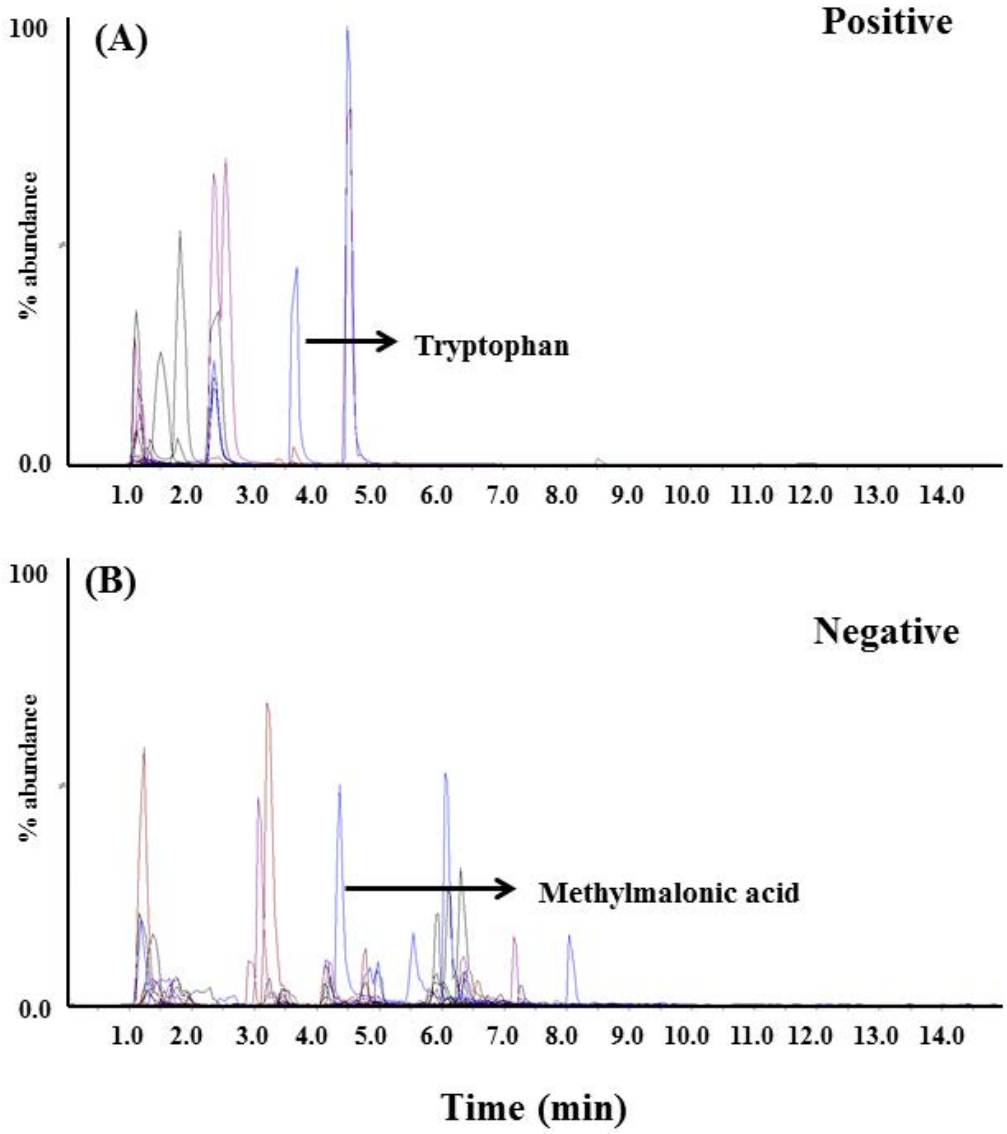

Figure 0.2: Metabolite quantification: Overlay chromatogram of 225 metabolites (**A**) Tryptophan in positive ion mode and (**B**) Methylmalonic acid in negative ion mode.
(Each coloured peak represents the amount of metabolite in the analysed samples).

and the analyte area ratio (area analyte/area IS) versus its nominal concentration was analyzed for linearity. The mean Pearson correlation coefficient (R2), calculated over the course of several days was greater than 0.98 for all targeted metabolites (**Table A1.3)**. Dealing with the simultaneous analysis of different classes of metabolites, the chemical complexity, and molecular heterogeneity can pose technical challenges in analyzing them in a single assay. For example, compared to LC-MS/MS, analysis of volatile organic compounds with high separation efficiency is possible using GC-MS, while it has some limitations in analyzing medium and highly polar metabolites requiring derivatization which can introduce additional substances resulting into overlapping signals, degradation of unstable metabolites and column damage. On the other hand, in LC- MS/MS analysis of non-derivatized polar and high molecular weight metabolites is possible. Hence often there is a demand to combine various analytical methods to obtain the best results.(39)

3.4.2 Method validation

3.4.2.1 Specificity and sensitivity

The LC-MS/MS method specificity was evaluated, and showed no interfering peaks at the corresponding retention times for the extracted compounds from different biological matrices such as whole blood, serum, and tissue. The sensitivity of the method was evaluated as described before and the results were summarized in **Table A1.3.** The lower limit of quantification (LLOQ), values are the average of the lowest point in each valid linear calibration curve with the relative standard deviation (RSD) ranging from 80 to 120%. Some compounds were below the values are the average of the lowest point in each valid linear calibration curve with the relative standard deviation (RSD) ranging from 80 to 120%. Some compounds were below the LLOQ, indicating that they could be detected in low concentration but could not be quantified. Sensitivity for each metabolite varied over wide ranges, as it depends on the compound polarity, fragmentation and ionization pattern and

post-source chemical stability. Under the optimum chromatographic conditions, peaks of the targeted metabolites were separated at retention times ranging from 0.73 to 11 min. Most of the metabolites eluted within 6 min except for sodium glycodeoxycholate and tauroursodeoxycholate which eluted soon after 11 min of retaining in the column.

3.4.2.2 Intra and inter-day accuracy and precision

Inter and intraday precision and accuracy were evaluated for the listed metabolites at three levels of QC samples; low, medium, and high concentration. The inter-day variability was less than 20% and inter-day accuracy ranged from 80 to 120% (**Table A1.4**) for the QCs, which meet the criteria of a validated bio analytical method to be utilized routinely for quantitative metabolomic profiling.

3.4.2.3 Stability

The stability of targeted compounds was studied under different preparation and acquisition conditions using the low, medium and high levels of the QC samples. It was calculated relative to freshly prepared samples, stored in liquid nitrogen as summarized in **Table A1.5.** The stability of some compounds showed a slight reduction after 6h but was very poor after 24 h, bench top storage (data not shown). Some compounds were unstable compared to the internal standard, which supports the need for having an isotopically labeled analog for each analyte. In general, the stability of most of the targeted metabolites under the operational conditions ranged between 60 and 150%, except under RT (6 h on the bench top) when it dropped below 60%. These stability ranges are considered valid for reporting because the area of LLOQ, as far as the method is concerned, is far above the noise and the RSD ranges between 80 and 120%. For DBS samples, metabolites long-term stability was evaluated relative to freshly prepared samples and is summarized in **Table A1.6**. Most metabolites including amino acids and carbohydrates were stable with minimal variance when analyzed

after 2 and 4 months. Itaconic acid, inosine 5'-diphosphate, L-carnitine, N-acetylspermine and 2-oxoglutaric acid showed no change when reanalyzed, whereas homocitrulline and sodium taurodeoxycholate showed significantly reduced stability.

3.4.2.4 Recovery

Recovery of the selected labeled internal standards (n=24) was used to evaluate the extraction recovery, where most of them were above 60% as summarized in **Table A1.7** except for methionine-d3, arginine-d7, and valine-d8.Overall the recovery data ranged between 54% & 27%.

3.4.3 Application of metabolomics analysis to biological samples

The metabolites in a biological sample are complex and have different chemo-physical properties, such as hydrophobicity, acidity, charge, pKa, size (1, 40) and the efficiency of extraction also varies depending on the metabolite itself. Hence, simultaneous separation of all the metabolites in a single comprehensive assay is challenging, (13, 41) and the metabolic profile is very sensitive to the biological matrix. The library of 225 targeted metabolites in this study are clinically relevant to many biochemical genetic diseases and are directly involved in different biochemical pathways that regulate a variety of important functions in the body. Examples of these pathways include metabolism of nucleotides, lactate/pyruvate, nicotinate and nicotinamide, biotin, glutathione, steroids, amino acids, polyols, vitamins, mitochondrial fatty acids oxidation and electron transport, glycolysis and pentose phosphate intermediates, hexosamine biosynthesis, malate-aspartate shuttle, as well as the "cancer related" Warburg effects. To evaluate the organ-specific metabolomic profile, tissue samples were collected from several organs (kidney, brain, heart, liver, skeletal muscle) of five male SD rats. As displayed in **Error! Reference source not found.A**, t he overall metabolite levels varied but was highest in the kidney tissue (35%) in comparison to the liver (25%), brain (17%), heart (15%) and muscle tissue (8%) for the same amount of tissue. Although the rat tissues were collected without perfusion, it is interesting to note the higher levels

of metabolites in the kidney which is probably related to its physiological function in excreting various compounds intermediary to metabolism and being the recipient of almost one-quarter of the total cardiac output.(42, 43) ANOVA analysis was performed for statistical (p-value) and fold change (FC), a significant set of metabolites in each tissue. The PCA score plot showed clear cluster differentiation among the five tissues (**Error! Reference source not found.B)**, which reflects t he reproducibility and the sensitivity of the method to detect the metabolic differential perturbation among the different tissue samples.

Pathway analysis of the significantly enriched metabolites was performed and **(Error! Reference s ource not found.C)** shows significant perturbation in pathways, such as the metabolism of nucleotides, nicotinate and the amino acids (alanine, aspartate and glutamate). The level of metabolic perturbations mainly follows the unique physiology of the organ or tissue. As shown in the heat map (**Error! Reference source not found.D**), cluster analysis shows unique metabolic s ignatures for the various tissues. For example, in contrast to the three other tissues studied, both skeletal and cardiac muscle showed perturbed levels of several nucleotides, creatine, and some hexoses. In the murine brain, creatinine and NANA (N- acetylneuraminic acid) was elevated relative to liver and kidney. The unusual dicarboxylic acid, itaconic acid (3-methylenesuccinic acid) was most abundant in murine brain, and to a much lower extent in skeletal muscle. Normally, decarboxylation of aconitate by aconitate decarboxylase (ACOD1) results in the formation of small quantities of itaconic acid. So far, no human patient with ACOD1 deficiency has been described. The “non-oxidative” pentose monophosphate shunt related metabolites, erythrose-4- phosphate, ribose-5-phosphate, fructose-6-phosphate, and sedoheptulose-7-phosphate seem to have perturbed patterns which correlate with known related human disorders. In particular, metabolism of nucleotides, nicotinate and amino acids. are most expressed in skeletal and cardiac muscles while sedoheptulose-7-phosphate is expressed primarily in brain and cardiac muscle. Transketolase (TKT, MIM # 617044) and transaldolase (TALDO1, MIM # 606003)

deficiencies are rare autosomal recessive disorders involving this pathway and are clinically characterized by congenital heart disease, facial dysmorphism, short stature and elevated plasma and urinary polyols

However, healthy neurological development, hepatosplenomegaly, and bone marrow dysfunction are features of TALDO1 deficiency, unlike TKT deficiency which had been associated with neuropsychiatric abnormalities. (44-46) Taken together; these observations suggest that accumulation of these pentose sugars may be causing cardiac dysmorphogenesis while the accumulation of sedoheptulose-7- phosphate, in particular, and may be neurotoxic. The polyamines, putrescine, spermidine, and spermine are multifunctional polycationic aliphatic amines that are involved in multiple vital cellular processes through translation initiation. Spermidine synthase (SRM, MIM # 182891) and spermine synthase (SMS, MIM # 300105) are responsible for the synthesis of spermine from putrescine through the generation of spermidine. Polyamine catabolism utilizes the rate-limiting enzyme SAT1 (spermidine/ spermine N (1) acetyltransferase 1) which has a higher affinity for spermidine than spermine and SAT2 (spermidine/spermine N (1) acetyltransferase 2), which has an equal affinity for both substrates.

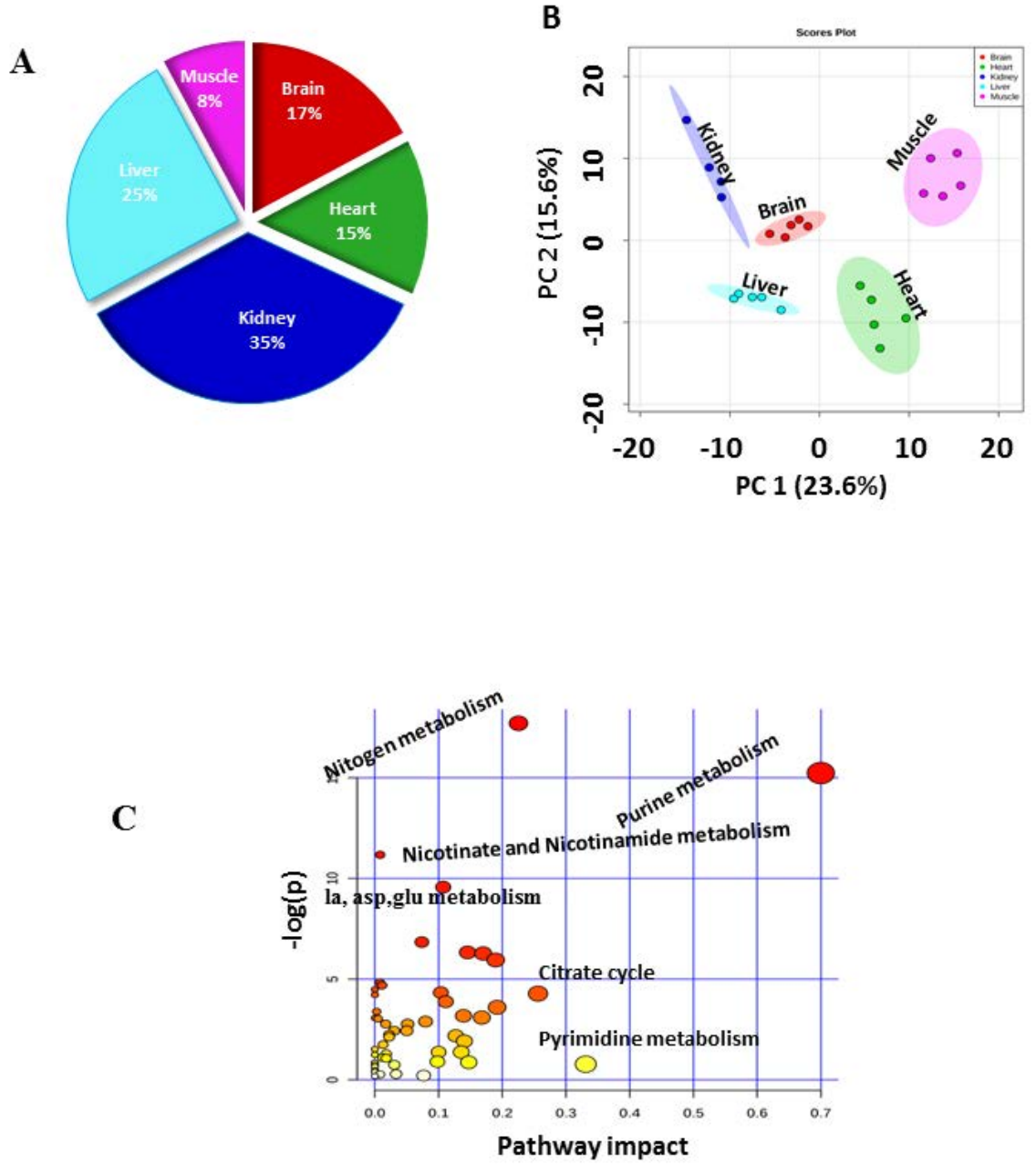

Figure 0.3 : Targeted metabolomic analysis of normal murine tissues. (**A**) Distribution of 225 targeted metabolites extracted from five different types of murine tissues (brain, heart, kidney, liver, muscle). (**B**) Principal component analysis (PCA) score plot of the five different murine tissues.(**C**) Analysis of significantly enriched pathways among the five tissue samples.

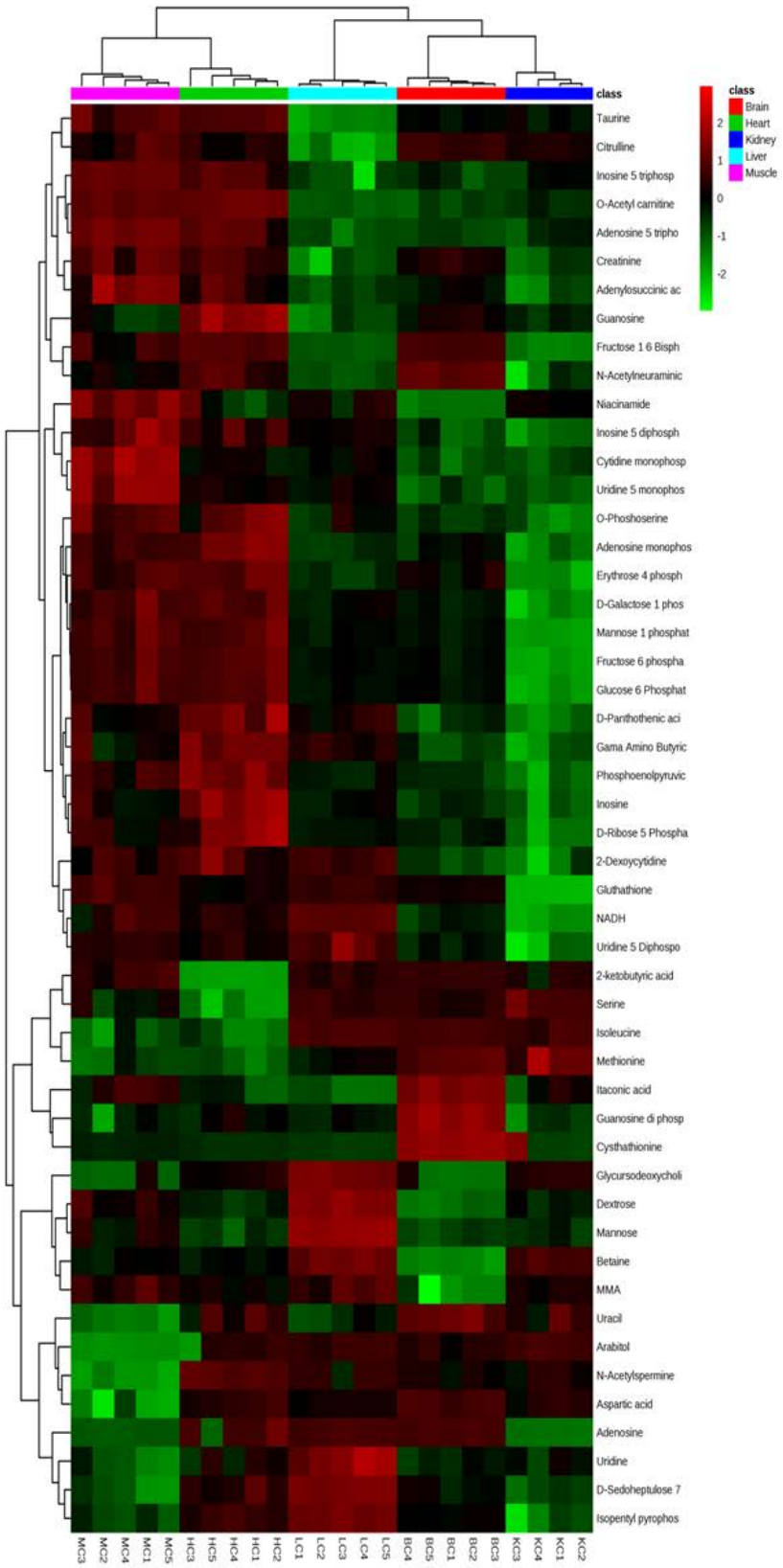

Figure 3.3(D) Heat map analysis of significantly enriched metabolites in 5 murine tissues (muscle, heart, liver, brain and kidney)

Levels of N-acetylspermine were highest in rat cardiac muscle, liver, and the least in skeletal muscle while putrescine and spermine were probably depleted. (**Error! Reference source not found.D**) (47) Recessive mutations in *SMS*, an X-linked gene, cause Snyder-Robinson mental retardation syndrome, which is characterized by developmental delay, facial dysmorphism and skeletal abnormalities.(48)To study the role of various biological matrices on the metabolic profile, whole blood samples from healthy individuals were collected and divided as blood, serum, and DBS. The metabolites levels were comparable in serum and whole blood as shown in the **Figure 0.4A**, but slightly higher in serum. Although both serum and plasma are commonly used to explore biomarkers in metabolomics studies, several studies have demonstrated that most metabolites are more stable and well expressed in serum.(49, 50) Dried blood spots showed the maximum metabolic level of analytes included in this panel, as compared to serum and blood samples, which is supported by other studies, where the largest number of metabolites was quantifiable and stable in DBS (at -80^{o}C under dry conditions).(51, 52) ANOVA analysis of metabolites in the three matrices (**Figure 0.4B**) showed high levels of 3-phosphoglycerate (DBS >serum> blood). Distinct cluster separation among the three matrices reflects the sensitivity of the method as shown in **Figure 0.4C**. Among the three matrices, pathway analysis of the significantly expressed metabolites shows amino acids metabolism to be most significantly perturbed (**Figure 0.4D)**. The healthy control DBS samples (n=200) included in the study were selected randomly from the NBS platform after they had been confirmed to be negative for any of the 17 inborn errors of metabolism that we target routinely in the Saudi newborn screening program.

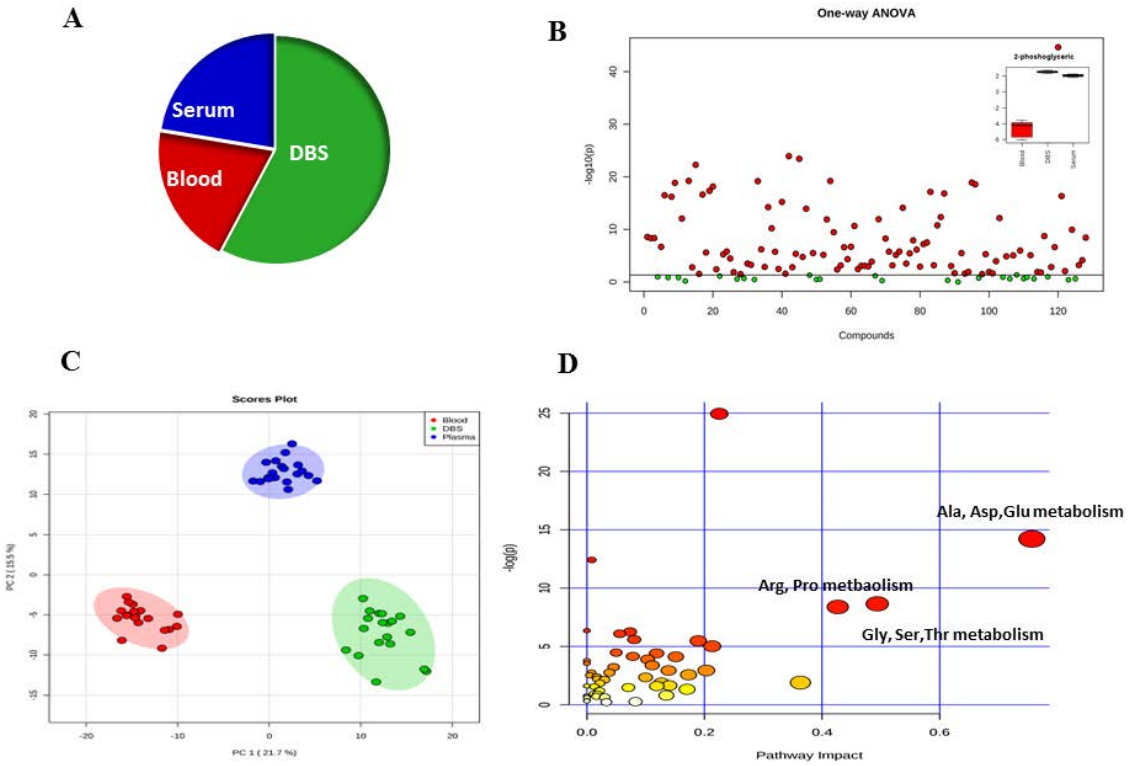

Figure 0.4:(A) Distribution of metabolites expression in various matrix sources. **(B)** One way Anova comparison of differential expression of metabolites between serum, blood and DBS samples. Inset shows expression profile of 3-phosphphoglycerate (DBS>serum>blood). (**C**) PCA analysis separates samples based on their matrix. (**D**) Pathway analysis of significantly enriched metabolites among the three matrices

These samples were then analyzed on this targeted metabolomics panel to study the technical reproducibility of the method, and to build a reference range for the analytes seen in the healthy newborn population. The reference ranges for the detected metabolites in DBS were calculated using non- parametric percentile method 95% CI, in this study was (2153.5 ± 42.4 nM), and for methionine 197.26 ± 5.39, the reference ranges for the rest of analytes are summarized in Error! Reference source not found.

3.4.3.1 Inborn errors of metabolism (IEMs)

In IEMs metabolomic profiling, using multiple biological matrices may represent the metabolic perturbations associated with these diseases.(7, 25) Early and accurate diagnosis of many of these disorders has proven to be critical in their management. In our study, samples from patients with known IEMs (n ¼ 56) were processed using this analytical platform, and the data were subjected to statistical analysis. Besides the focused diagnostic key MS/MS based markers used routinely and summarized in **Table 0.1,** several other metabolites were found to be differentially expressed in these patients compared to ctrl samples such as betaine, succinic acid, niacinamide, fructose 1,6-bisphopshate (Error! Reference source not found.). All of these samples were diagnosed based on the gold standard key biomarkers such as branched-chain amino acids in patients with MSUD, propionyl/C3-carnitine and methylmalonic acid in MMA, tetradecadienoyl/C14:1 carnitine for VLCAD, glutarylcarnitine and 3-hydroxyglutaric aciduria for glutaric aciduria type 1 patients, and phenylalanine for PKU patients. As shown in Error! Reference source not found., several sugars (arabinose, ribulose, dextrose, fructose and mannose) appeared to be overexpressed, in all studied groups of inborn errors of metabolism more so in the disorders of mitochondrial metabolism (VLCAD and GA2 deficiency) compared to ctrls. Biotin deficiency was detected in patients with various inborn errors of metabolism suggesting dietary deficiency, the correction of which may have a positive clinical impact on these patients. Betaine (trimethylglycine) is a

dietary supplement abundantly present in seafood, plants (such as wheat germ, bran, spinach), and is important as an osmolyte that protects cells under stress and as a methyl group donor significant for the hepatic methionine cycle.(53, 54) Betaine supplementation appears to be adequate in these patients as shown in these metabolomics profiles.

3.4.3.1.1 Methylmalonic acidemia (MMA) & propionic acidemia (PA)

Methylmalonic aciduria is a biochemically, molecularly and clinically heterogeneous disorder which is caused by a deficiency of methylmalonyl CoA mutase (MCM), defects in the transport or synthesis of its cofactor, adenosyl-cobalamin (cblA, cblB, cblC, cblF, cblD and cblX), deficiency of methylmalonyl-CoA epimerase (MCEE). The biochemical hallmarks of MMA include high plasma propionyl/C3-carnitine and elevated urinary excretion of MMA and methylcitrate. On the other hand, elevated plasma propionyl carnitine, urine methylcitrate, propionic acid and propionylglycine characterize the metabolic profile of patients with propionic acidemia as shown in **Error! Reference source not found.A** Secondary h yperglycinemia occurs in both disorders as well as in several other organic acidemias. For proper diagnosis and management, metabolic profiling in organic academia is essential, as they are almost clinically indistinguishable.

The overall incidence in the Western population ranges from 1:48,000 to 1:51,000 births for MMA, (55) and from 1:50,000 to 1:500,000 births for PA, whereas in Saudi Arabia, the incidence is much higher at 1 in 2,000 to 5,000 live births.(56) Clinical symptoms are non-specific and start as early as second day of life with deteriorating clinical conditions like vomiting, dehydration, weight loss, temperature instability, neurological involvement with muscular hypo of hypertonia, lethargy progressing to coma and seizures. Laboratory findings include metabolic acidosis and ketosis.(56, 57) Newborn screening for MMA and PA is possible using propionylcarnitine and methionine. (58, 59)

As shown in Error! Reference source not found., MMA (n=6) and PA (n=2) displayed distinct m etabolomics profiles with minimal overlap including hyperglycinemia and related elevated glutamine levels. Interestingly, spermine appears to be markedly reduced, in MMA compared to PA suggesting a preferential toxicity to the synthetic polyamine pathway in such patients. Another intriguing observation is the lower level of ethanolamine in MMA patients (as well as patients with PKU, GA2, VLCADD) in comparison to PA. Ethanolamine is produced via the decarboxylation of serine and is essential for the synthesis of phospholipids. In the MMA group of samples (n= 6), MMA was significantly elevated as expected as in **Error! Reference s ource not found.B,** whereas **Error! Reference source not found.C** summarizes some of the noted biochemical perturbations in connection with MMA metabolism.

3.4.3.1.2 Phenylketonuria (PKU)

Classical phenylketonuria is caused by a deficiency of hepatic phenylalanine hydroxylase (PAH) and results in the accumulation of phenylalanine (Phe) and its catabolites (phenylpyruvate, phenyllactate, and phenylacetate). Loss of PAH activity results in increased concentration of phenylalanine in blood and toxic concentrations in the brain. Untreated PKU is associated with progressive intellectual impairment, along with other symptoms like eczematous rash, autism, seizures and motor deficits. As the child grows up, other developmental problems, aberrant behavior and psychiatric symptoms become evident. Enzyme deficiency can lead to mild hyperphenylalaninemia, mild phenylketonuria, and classic phenylketonuria. Classic phenylketonuria is a result of complete deficiency of phenylalanine hydroxylase activity, leading to irreversible intellectual disability, whereas mild hyperphenylalaninemia and mild phenylketonuria are linked to a lower risk of impaired cognitive development if left untreated. In Europe, the prevalence of PKU is 1: 10,000 live births,(60) whereas in Saudi Arabia the prevalence ranges from 0.005% to 0.0167%. The highest prevalence was reported in Turkey at 0.0167%, whereas the lowest prevalence was

reported in the UAE, 0.005%.(61) Due to well advanced newborn screening (NBS) programs reported in many developed countries, classical phenylketonuria are rarely reported , as PKU can be diagnosed readily in blood specimens collected by heel-prick from newborns.(62) Early diagnosis and prompt interventions can help avoid mental disability and a lifelong Phe restricted diet is the basis of current management of PKU.(63)

In the PKU group of samples (n=15), Phe level was significantly elevated as expected and shown in **Error! Reference source not found.C**.As shown in **Error! Reference source not found.A** Hy perphenylalaninemia causes increased production and excretion of phenylpyruvic acid, an inhibitor of the mitochondrial enzyme pyruvate carboxylase, a key regulatory enzyme involved in gluconeogenesis, lipogenesis, and neurotransmitter synthesis, which converts pyruvate to oxaloacetate.(64)As a result, most glycolysis intermediates such as PEP, F-1, 6-BP are up-regulated along with down regulation of lactate as shown in **Error! Reference source not found.B.** This perturbations leads to the accumulation of succinate, carnitine, and acetylcarnitine and reduces the conversion of acetyl-CoA through oxaloacetate to citrate (**Error! Reference source not found.D**).Hydroxyproline was found to be up-regulated in PKU p atients suggesting altered bone metabolism. Several studies reported low bone mineral density (BMD) in patients with PKU, which is formula independent. (65, 66)

3.4.3.1.3 Hereditary tyrosinemias (Type-1 and type 2)

Tyrosinemia type-1 is caused by a deficiency of fumarylacetoacetate hydrolase (FAH), the last enzyme involved in the phenylalanine/tyrosine degradation pathway while tyrosinemia type 2 results from a deficiency of tyrosine transaminase (TAT).(67)These two tyrosinemia syndromes are quite distinct, both clinically and biochemically as succinylacetone (4, 6-dioxoheptanoic acid) accumulates in patients with type 1 only.

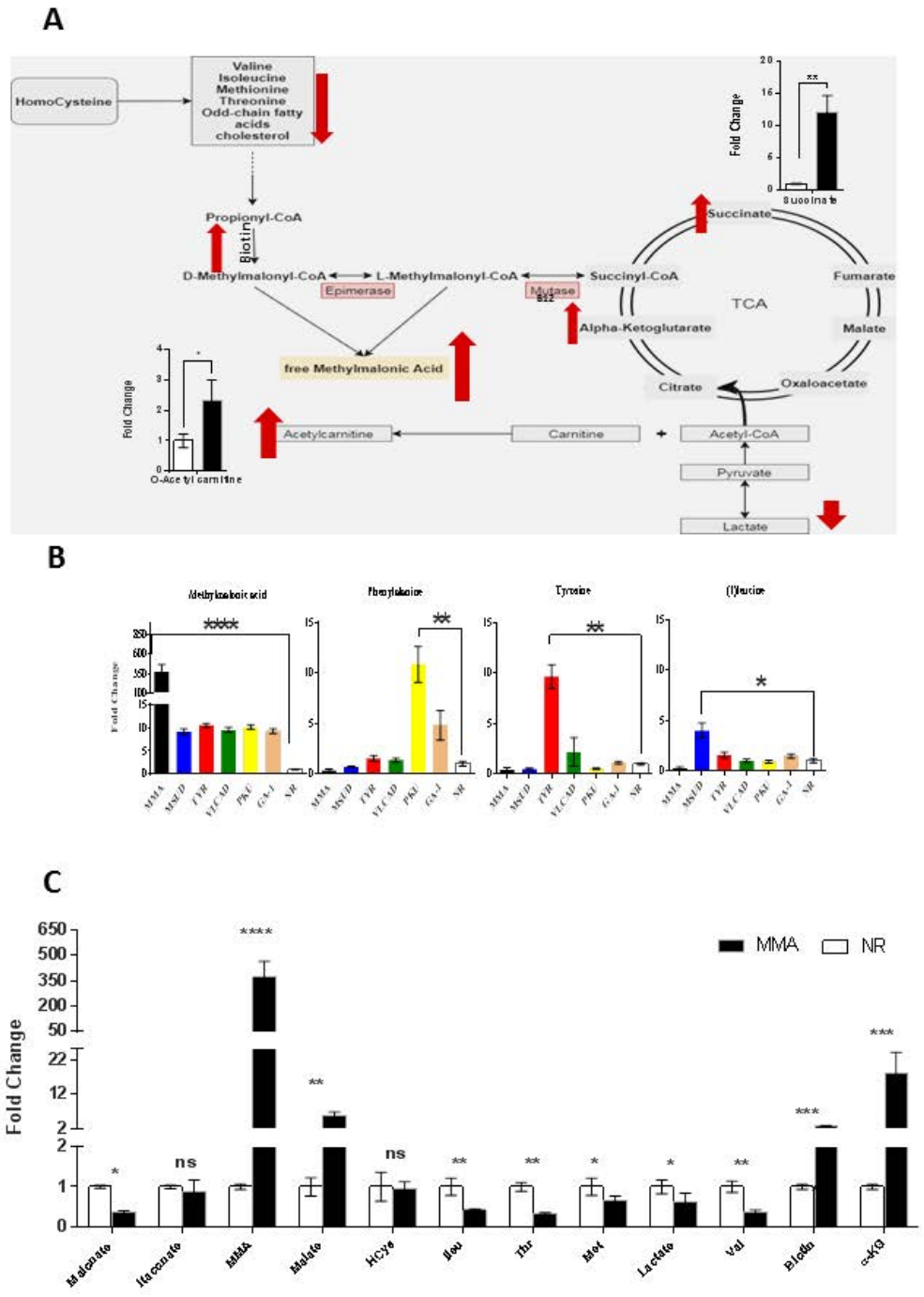

Figure 0.5: Targeted metabolomic profiling in DBS samples of patients with various known inborn errors of metabolism.(A) Metabolic perturbations in methylmalonic aciduria and (B) related pathways, where MMA is associated with multiple significant metabolic abnormalities includingbiotin deficiencyand hyperhomocysteinemia. (C) Characteristic metabolite profiles in selected inborn errors of metabolism. * denotes statistical significance.

(Abbreviations: Methylmalonic acidemia- MMA, Maple syrup urine disease- MSUD, Very ling chain fatty acid dehydrogenase deficiency- VLCAD, Glutaric type-1- GA-1, Not remarkable- NR.)

The disease is characterized by progressive liver disease and renal tubular dysfunction that leads to hypophosphatemic rickets, cirrhosis and hepatocellular carcinoma.(68) Prevalence estimates from 1 in 250,000 births. (69) The presentation of the disease may occur in infancy, childhood or as adults and age of presentation broadly correlates with severity of the disease .(70) In 10 tyrosinemia samples (Tyr1=7, Tyr2=3), the metabolomics profiles were quite distinguishable from the control group (**Error! Reference source not found.C, E**) but not from e ach other presumably because succinylacetone excretion was controlled under therapy. Tyrosinemia groups of samples when compared to controls (**Figure. A2.6**), Tyr and Phe, nucleotides, carnitine and betaine were upregulated while sugars, niacinamide, progesterone as well as amino acids were down-regulated. While both disorders share partially the same metabolic pathway, additional dietary and therapeutic interventions likely contributed to this pattern (**Error! Reference source not found.E** and Error! Reference source not found.**)**.

3.4.3.1.4 Maple syrup urine disease (MSUD)

MSUD is an aminoacidopathy caused by recessive mutations in one of the lipoic acid dependent enzymes: branched chain keto acid dehydrogenase-E1 alpha polypeptide (BCKDHA); Branched Chain Keto Acid Dehydrogenase E1 Subunit alpha (BCKDHA); Branched Chain Keto Acid Dehydrogenase E1 Subunit beta (BCKDHB); dihydrolipoamide branched chain transacylase (DBT), responsible for the catabolism of the branched-chain amino acids leucine, isoleucine, and valine. Accumulation of these three amino acids and their corresponding ketoacids leads to encephalopathy and progressive neurodegeneration in untreated infants.

Patients with severe forms of MSUD develop metabolic decompensation and encephalopathy within the first weeks of life and may lead to death if left untreated.(71) Continuous metabolic control of these patients is possible by DBS monitoring programs, (72) because if

the disease progress convulsion, hypoglycemia, coma and systemic failure may occur. Hence, early detection and treatment is essential for long-term disabilities. Restricted dietary management with low BCAAs (mainly leucine) has to be followed in these patients or the ultimate treatment is liver transplantation.(73) As expected, in our group (n=10) of patients with MSUD, the key diagnostic markers leucine, isoleucine, and valine were elevated when compared to the ctrls (**Error! Reference source not found.B** and **Figure A2.6**). In addition, p anthothenic acid and 4-hydroxyproline were up regulated, whereas progesterone, niacinamide, and ethanolamine were down regulated.

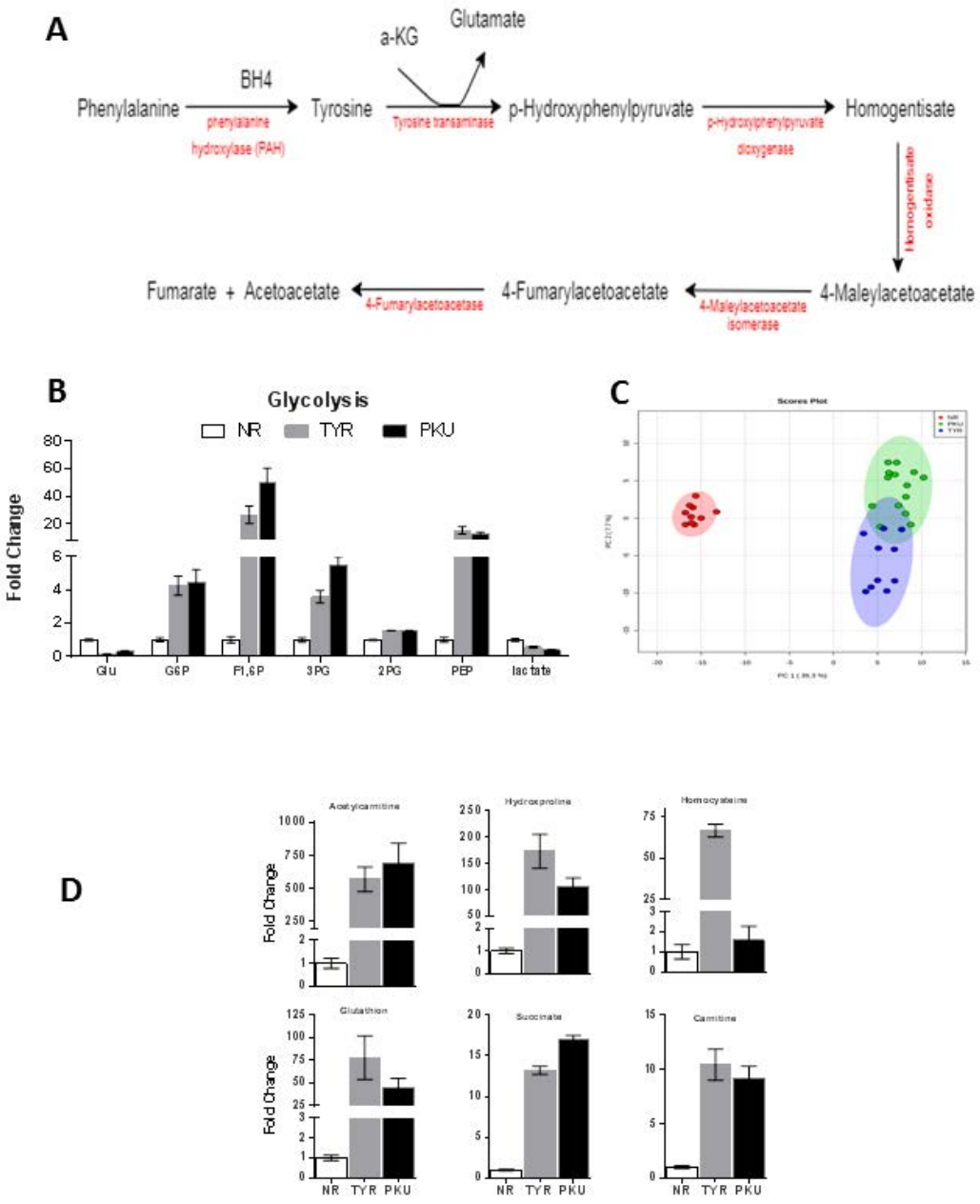

Figure 0.6: Comparison of targeted metabolomic profiles in patients with phenylketonuria (PKU) vs. tyrosinemia type 1 (TYR-1).(**A**) Metabolic pathway of phenylalanine and tyrosine metabolism. (**B**) Several glycolytic intermediates are almost equally overexpressed in both PKU and TYR-1 patients. (**C**) PCA score plots for PKU (n15) and TYR-1 (n=10) vs control subjects show clear separation of the three groups with partial overlap between the PKU and TYR1 subjects. (**D**) Significant perturbations in amino acids (especially homocysteine and glutathione) and carnitine metabolism were detected in both disorders.

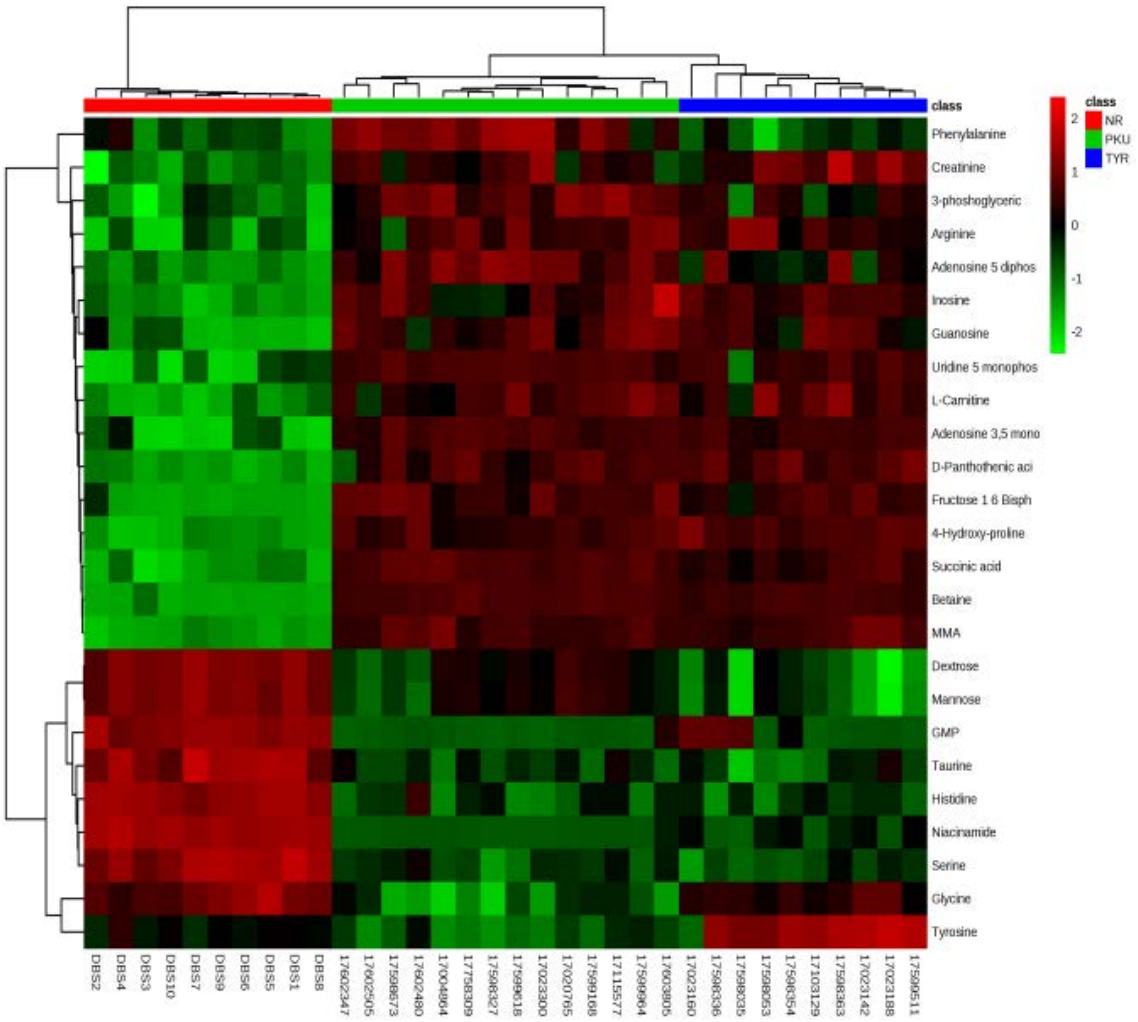

Figure 3.6E: Heat map analysis of enriched metabolites in PKU and TYR-1 shows distinct metabolomic profiles compared to healthy controls.

3.4.3.1.5 Very-long-chain acyl-coenzyme A dehydrogenase deficiency (VLCADD)

VLCADD, is an autosomal recessive disorder with a prevalence of about 1:85,000 (74) and results in impaired mitochondrial b-oxidation of long chain fatty acids. It presents with a disease specific acylcarnitine profile with elevated C14:1-carnitine as a unique blood marker. (75) Classically, two forms of VLCADD have been described: an early-onset, severe form which, if unrecognized and undiagnosed, may lead to extreme weakness of the heart muscles (cardiomyopathy) and may be life-threatening, and a later-onset, milder form that is characterized by low blood sugar (hypoglycemia). (76) Treatment consists in avoidance of fasting, prompt treatment of fever, other illnesses and supplementation of a diet low in long chain fatty acids and medium chain triglyceride.(77) In patients with secondary carnitine deficiency, L-Carnitine at low doses is recommended followed by monitoring including annual physical examination .(78) VLCADD can be detected through newborn screening programs, but there are chances to miss some cases as acylcarnitine profile can become normal during anabolic conditions. (79)(80) In the ten profiled samples, niacinamide, glycerol, ethanolamine were significantly under-expressed (Error! Reference source not found.).

3.4.3.1.6 Glutaric acidemias: GA-1 and GA-2.

Glutaric acidemias are autosomal recessive neurometabolic disorders with mutations in glutaryl-CoA dehydrogenase (GCDH) causing type 1 (GA-1) and mutations in *ETFA, ETFB* and Electron transfer flavoprotein dehydrogenase (*ETFDH)* causing type 2 (GA-2). Detection of glutarylcarnitine (C5DC) by tandem mass spectrometry is the primary biomarker used in newborn screening for for GA-1.(81) In contrast, elevation of plasma C4/butyrylcarnitine and C5/ isovalerylcarnitine mark the acylcarnitine profiles of patients with GA-2 (MADD/multiple acyl-CoA dehydrogenase deficiencies).

GA-1 patients have a deficiency of glutaryl-CoA dehydrogenase enzyme (82) that is required to break down the amino acids lysine, hydroxylysine, and tryptophan and prevalence of GA-1 is 1 in 56000. (83) Accumulation of these amino acids can cause cerebral atrophy and brain damage,(84) characterized by macrocephaly at birth or shortly after, dystonia, and seizures at the first episode. (85) Treatment includes carnitine supplementation, diet restricted in amino acids capable of producing glutaric acid, and prompt treatment of occurring illnesses.(86) GA-II represent three types of clinical phenotypes: the neonatal form with congenital anomalies, the neonatal form without congenital anomalies; and the late onset form with myopathic phenotype.(87) MADD is a disorder of fatty acid and amino acid oxidation and is a clinically heterogeneous disorder ranging from a severe neonatal presentation with metabolic acidosis, cardiomyopathy and liver disease, to a mild childhood/adult disease with episodic metabolic decompensation, muscle weakness, and respiratory failure.(88) These patients are advised a protein- and fat-restricted, carbohydrate-rich diet as well as riboflavin, glycine, and L-carnitine supplementation. (88)

The metabolomic profiles of GA-1 (n= 2) and GA-2 (n= 1) showed upregulation of pantothenic acid, betaine, 3-phosphoglycerate, inosine and hypoxanthine in both GA-1 and GA-2 samples, whereas several sugars such as dextrose, ribose, and mannose were downregulated. Interestingly, spermine and (to a lower extent) alanine were preferentially downregulated in GA-2 but not in GA-1 (Error! Reference source not found.). This may c ontribute to the pathogenesis of hypoglycemia in these disorders.

In the last two decades, LC-MS/MS has become the main technique for analyzing DBS in newborn screening programs worldwide. To achieve maximum sensitivity, clinical laboratories apply conservative cut-off values resulting in decreased test specificity and consequently a higher rate of false-positive results. Consequently, second tier tests were developed, to improve the specificity of screening processes.(32, 33) The potential advantage

of "comprehensive" untargeted metabolomics over traditional limited targeted analysis depends on its scope of detection providing additional data that currently requires multiple clinical tests, including targeted panel assays for amino acids, acylcarnitines, organic acids, purines, pyrimidines targeted panel assays for amino acids, acylcarnitines, organic acids, purines, pyrimidines, acylglycines and others.

The described metabolomics method, although is a pilot study with a limited 225 analytes, seems to be a promising assay in individuals at risk of having one of the common inborn errors of metabolism and is potentially clinically useful. The large number of analytes targeted in this single assay in three commonly used matrices, coupled with the reference ranges and average values in the control subjects may help facilitate identifying the optimal matrix for various analytes. For individuals with metabolic disorders caused by defects in the metabolism of large molecules such as lipids and glycosaminoglycans (GAGs), additional studies using lipidomics, for example will be needed.

In summary, there is a strong clinical need to develop and validate quantitative methods capable of measuring the largest number of metabolites subject to wide and rapid dynamic changes present in a biological system.(15) This is particularly challenging since several important factors such as metabolite dilution, stability, low abundance, sample matrix, concentration and availability of internal standards may significantly impact the ability to quantify these analytes. In the present study, the effect of sample matrix on the expression of profiles of a large set of clinically oriented metabolites was demonstrated which may help to guide the choice of the optimal sample type and matrix in which it is collected. The developed method showed capability to detect common inborn errors of metabolism. In chapter 4, same approach was used and seven metabolites were positively identified in binary comparisons between DOCK8- deficient and AD patients. Aspartic acid and 3-hydroxyanthranillic acid were up-regulated in DOCK8 compared to AD, whereas

hypotaurine is down-regulated compared to ctrl. Leucyl-phenylalanine, and glycyl-phenylalanine were down-regulated in DOCK8 compared to AD, guanosine was upregulated in DOCK8 when compared to ctrl and 2- aminooctanoic acid was up-regulated in AD compared to ctrl.

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3.6 Chapter 3 summary

- The aim of this study was to develop a metabolomics method to analyze DOCK8 and AD patients for biomarker discovery.
- A comprehensive metabolomics panel was built to accommodate 225 metabolites within a short run time of 15 minutes for both polarity modes (negative and positive).
- The method was validated in regards to specificity, sensitivity, intraday accuracy and precision, stability and recovery as per US FDA regulations.
- The developed method includes a list of optimized MS/MS parameters and can be potentially useful as a screening tool for different autoimmune and other disease categories.
- This methodology can also be used in three different matrices (blood, serum and DBS), and is also coupled with the reference ranges and average values in the control subjects.

CHAPTER 4: METABOLOMICS DISTINGUISHES DOCK8 DEFICIENCY AND ATOPIC DERMATITIS: TOWARDS A BIOMARKER DISCOVERY.

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4.1 Introduction

DOCK8 deficiency is caused by loss of function mutations in the dedicator of cytokinesis 8 (*DOCK8*) gene,(1, 2) characterized by susceptibility to sinopulmonary infections, atopic eczema, asthma, food allergies, severe viral infections, increased incidence of malignancy leading to premature death. (2, 3)DOCK8 is a cytoskeletal protein which contains two related conserved protein domains DHR1 and DHR2 with bi-allelic *DOCK8* mutations having both been reported with frequent large deletions and point mutations, leading to protein loss of function. (2, 4)It is highly expressed in the immune system especially in lymphocytes but is also expressed in placenta, kidney, lung and pancreas.(5) DOCK8-deficient patients develop atopic dermatitis, asthma, and severe allergies to food and environmental antigens in early infancy.(6) In addition, chronic viral infections are also distinctive features with the common pathogens being herpes simplex virus (HSV), human papillomavirus (HPV) molluscum contagiosum virus (MCV) and varicella-zoster virus (VZV). In general, all DOCK8-deficient patients are susceptible to recurrent sinopulmonary infections caused by a wide variety of pathogens including *Streptococcu spneumoniae, Haemophilus influenzae, Pneumocystis jirovecii, Histoplasma capsulatum and Legionella pneumophila.* (7)

Atopic dermatitis (AD) or eczema is a prevalent pediatric chronic inflammatory skin disease, and specific food-allergens and nutrients are closely related to the development and severity of this disease. AD is characterized by intense pruritus and occurs primarily in infants and children, with approximately 70% of cases starting before the age of 5 years. The eczema classically involves the face, scalp and extensor surfaces of extremities. Impaired innate and adaptive immunity, environmental changes and alterations in genes involved in epidermal barrier functions contribute towards the clinical manifestations of this disease.(8) These patients are susceptible to superficial infections with *Staphylococcus aureus*, but invasive infections rarely occur in AD, unlike DOCK8-deficient patients. Janssen et.al (2014) has

shown flow cytometry biomarkers distinguishing DOCK8 from AD.(9) Whereas Boos et.al(2014) showed that DOCK8 mutation plays an important role in food allergies, and different IgE sensitization patterns in HIES and AD patients.(6) Treatment of AD is directed mainly towards prevention and management of infection and immunomodulation to control the associated rash and pruritus. Topical corticosteroids, systemic antibiotics, and antifungal agents are used for both prophylactic and symptomatic treatment in conjunction with topical therapy. Atopic dermatitis and DOCK8 deficiencies share similar clinical symptoms including eczema, eosinophilia, and characteristic elevated levels of serum IgE.

As discussed in Chapter 1,Metabolomics is a rapidly growing and promising discipline enabling the quantification of the group of small molecules involved in intermediary metabolism encoded by genomic DNA. Over the last decade, both targeted and untargeted metabolomics studies have identified several relevant biomarkers involved in complex clinical phenotypes in diverse biological systems. Significant environmental and clinical disturbances can be monitored at the metabolomic level by examining an array of different pathways that are crucial for cellular homeostasis.(10, 11) Since the metabolome is complex and very dynamic, newer and more reliable quantitative technologies have enabled the discovery of biomarkers specific enough to distinguish patients in various health states from healthy subjects.(12) Chemical isotope labeling liquid chromatography-mass spectrometry (CIL LC-MS) is a robust and emerging analytical platform used in biomarker discovery, where different labeling reagents are used to target functional groups based sub-metabolomes.(13, 14)

Apart from cytokine biomarkers capable of distinguishing DOCK8-deficient (6,9, 15) from patients (16) definitive metabolomics biomarkers have not been identified yet. Therefore, in-depth metabolomics technologies to study the metabolomics profiles of a cohort of patients with DOCK8-deficient and AD was studied to explore biomarkers that potentially reflect

disease pathogenesis and may contribute towards improved disease monitoring and ultimately novel clinical interventions. CIL LC-MS targeting the amine/phenol sub-metabolomes was used to find novel differentially expressed biomarkers in hereditary DOCK8-deficient and AD patient groups.

4.2 Aims

The aims of this study were as follows:

1. To employ CIL LC-MS to study the metabolomics profiles in DOCK8 and AD patients to find novel and differentially expressed biomarkers
2. To evaluate these biomarkers that potentially reflects disease pathogenesis and may contribute towards improved disease monitoring and ultimately novel clinical interventions.

4.3 Material and Methods:

4.3.1 Chemicals

The LC-MS grade reagents, including water, acetonitrile (ACN), methanol and formic acid, were purchased from Fisher Scientific (Ottawa, ON)and13C-dansyl chloride was available from the University of Alberta)(http://mcid.chem.ualberta.ca). For cell culture, Rosewell Park Memorial Institute (RPMI) medium, penicillin, glutamine was obtained from Sigma Chemicals, (St. Louis, MO), and Fetal bovine serum (FBS) from Gibco, Life Technologies, (Saint-Aubin, France)

4.3.2 Characteristics of the study population:

As discussed in chapter 2, the metabolomics study cohort for DOCK8-deficient and AD patients were same.(17) This study was approved by the Research Ethics Committee at the Office of Research Affairs, at King Faisal Specialist Hospital and Research Center (KFSHRC) (RAC No. 2160015).

4.3.3 Cell culture

Lymphoblastoid cell lines were obtained by transformation of peripheral blood mononuclear cells (PBMCs) as discussed in chapter 2.(17)

4.3.4 LC-MS

In this CIL LC-MS metabolomics workflow (**Error! Reference source not found.)**, each sample w as labeled by^{12}C-dansyl chloride (DnsCl), while a pooled sample was generated by mixing all individual samples then labeled by ^{13}C- DnsCl.(13) The ^{13}C-labeled pooled sample served as a reference for all the ^{12}C-labeled individual samples. Each sample was normalized prior to LC-MS analysis. LC-UV quantitation was performed to determine the total concentration of dansyl-labeled metabolites. Each ^{12}C-labeled sample was mixed with the same molar amount

of ^{13}C-labeled pooled sample and injected into LC-MS. All labeled metabolites were identified as peak pairs on mass spectra, and the peak area ratios were used for quantitative metabolomic analysis.

The serum and cell lysates processed samples were analyzed using a Thermo Fisher Scientific Dionex Ultimate 3000 UHPLC System (Sunnyvale, CA) linked to a Bruker Maxis II quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker, Billerica, MA). The LC column was an Agilent reversed phase Eclipse plus C18 column (2.1 mm × 10 cm, 1.8 μm particle size, 95 Å pore size), while the mobile phase A was 0.1% (v/v) formic acid in 5% (v/v) ACN, and solvent B was 0.1% (v/v) formic acid in acetonitrile. The LC gradient was: t = 0 min, 20% B; t = 3.5 min, 35% B; t = 18 min, 65% B; t = 21 min, 99% B; t = 34 min, 99% B, with a flow rate of 0.18 mL/ min. The MS conditions were as follows: polarity, positive; dry temperature, 230 °C; dry gas, 8 L/min; capillary voltage, 4500V; nebulizer, 1.0 bar; end plate offset, 500V; spectra rate, 1.0 Hz.

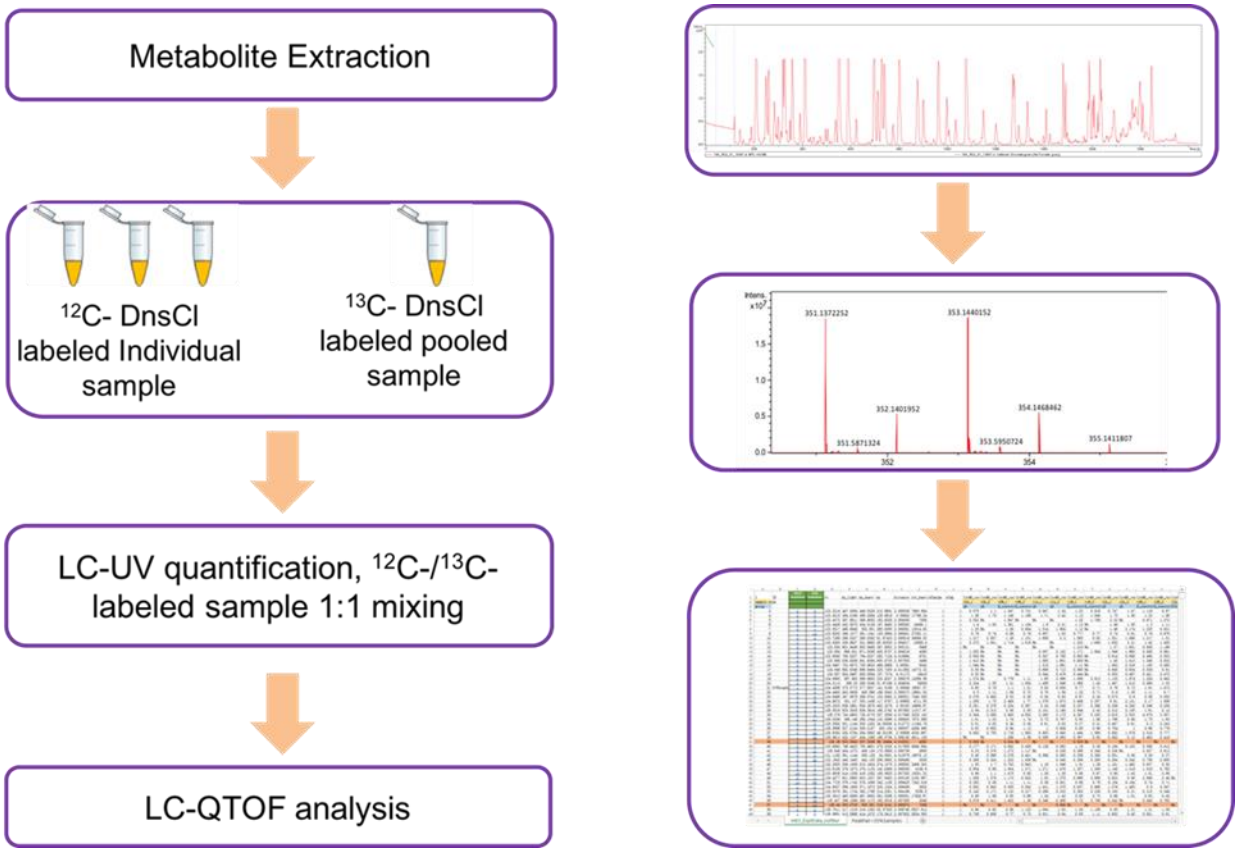


Figure 0.1: Flowchart of metabolomics workflow

4.3.5 Data collection, processing, and analysis

The LC-MS spectra were first converted to csv files by Bruker Daltonics Data Analysis 4.3 software. The peak pairs were extracted from csv files by IsoMS. Meanwhile the redundant pairs (e.g. those of Na^+, NH_3^+adduct ions and dimers) were filtered out.(18) All data generated from multiple runs were aligned together based on the peak's accurate mass and retention time. The missing values in the aligned file were filled by Zero fill software.(19) A univariate analysis (volcano plot) was performed for each binary comparison to identify significantly differentially expressed metabolites. A criterion of fold-change greater than 1.5 or less than 0.67 with q-value (false discovery rate) less than 0.05 was used. The q-value is calculated by R script based on p-value from a t-test. In the volcano plot, the x-axis represents the fold change (FC) between two comparison groups, and y-axis represents the p-value. The principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed using MetaboAnalyst (www.metaboanalyst.ca).(20) The metabolites were positively identified by searching against DnsID Library (www.mycompoundid.org) using retention time and accurate mass.(21) Putative identification was performed by searching accurate mass against My Compound ID library, which contains 8,021 known human metabolites and 375,809 predicted metabolites.(www.mycompoundid.org) (22)

Statistical analysis among the three groups was performed by Analysis of Variance (ANOVA) using post-hoc Tukey's method of analysis, with multiplicity adjusted p-values for each comparison. This method was chosen not only because of the unequal group sizes, among the experimental and the ctrl groups but also because it reduces the probability of making a type 1 error and supports testing of pairwise differences. Further analysis was performed on GraphPad Prism (version 6.0, Graph Pad software, LA Jolle, CA). The Receiver Operating Characteristic (ROC) curves were constructed using random forest

method MetaboAnalyst software version 3.0 (McGill University, Montreal, Canada) (http://www.metaboanalyst.ca) for global analysis. The raw data was normalized, transformed, and scaled by a median, log, and Pareto respectively, to make sure all the data are visualized under Gaussian distribution.

4.4 Results

4.4.1 Clinical characterizations of DOCK8-deficient and AD patients

As discussed in chapter 2, clinical and laboratory characteristics of the study cohorts are represented in

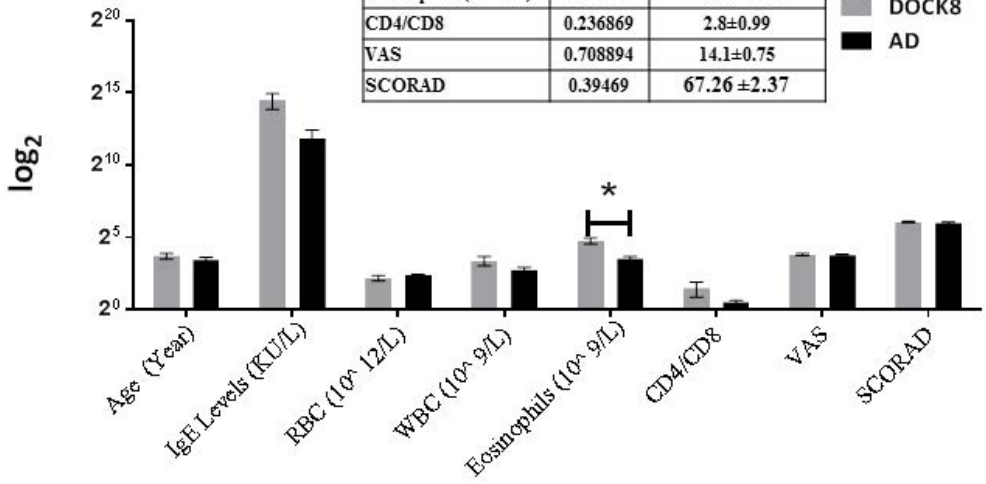
Table A1.9 The mean age of the DOCK8-deficient and AD cohort were13.2±5.9 and 10.8±1.4 years, respectively, where the Ctrl was 23± 1.03.Comparatively, the CD4/CD8 ratio in the DOCK8-deficient cohort (2.8 ± 0.99) was higher compared to the AD cohort (1.43±0.14) (

Table A1.9) (17)

Total IgE levels in both DOCK8-deficient and AD groups were elevated when compared to the ctrl group, with DOCK8-deficient patients showing significantly higher serum IgE levels (p-value <0.05) compared to AD patients and ctrls. (5-500KU/L) Eosinophil levels was seen to be significantly up- regulated in DOCK8-deficient patients compared to AD.(**Error! Reference source not found.A**) Among the DOCK8-deficient c ohort splicing mutations were the most common (46%), followed by missense mutations (27%), deletion mutations (20%) and stop codon mutations (7%). (**Error! Reference source not found.B)** (17)

A

Parameter	P value	Average±SEM
Age (Year)	0.354394	13.2±5.9
IgE Levels (KU/L)	0.043089	19817.30±4772.6
RBC (10^ 12/L)	0.228738	4.5±0.57
WBC (10^ 9/L)	0.162607	10.53±2.3
Eosinophils (10^ 9/L)	0.002506	27.29±4.02
CD4/CD8	0.236869	2.8±0.99
VAS	0.708894	14.1±0.75
SCORAD	0.39469	67.26 ±2.37



B

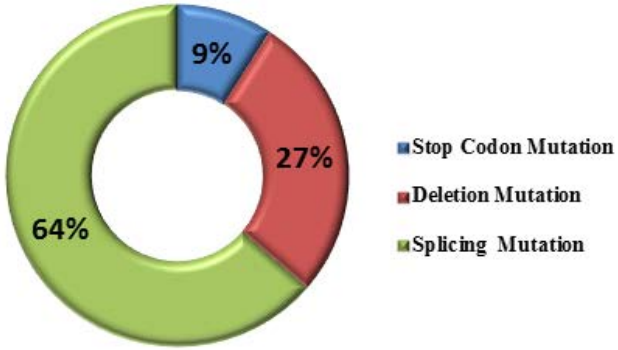


Figure 0.2: Analysis of clinical and laboratory parameters in DOCK8 deficient and atopic dermatitis (AD) patients. (A) Eosinophil counts are significantly different between the DOCK8 deficiency and AD groups but not the serum IgE levels.(B) Distribution of mutations in DOCK8 deficiency patients (One way ANOVA, Post hoc Tukey's method, ** p-value <0.001).

4.4.2 Metabolomics profiling

Pathway analysis **(Error! Reference source not found.A)** identified nitrogen (global) amino acid metabolism pathways to be the most perturbed, f ollowed by an amino acyl-tRNA biosynthesis when DOCK8 deficiency was compared with AD and Ctrl. The global metabolomics profiles was dissected in several binary analyses for a better understanding of the distinctive contribution of each gene in DOCK8 deficiency group compared either to AD or Ctrl groups. Partial least square discriminant analysis (PLS-DA) score plot demonstrates significant separation between the DOCK8 deficiency and Ctrl groups (**Error! Reference source not found.B)**. The univariate and volcano plot analyses were also performed and a t otal of 3442 metabolites features were detected, among them, a group of metabolites (n=481) was differentially expressed and visualized in the volcano plot **(Error! Reference source not found.C).** The cutoff p-value has a corresponding q-value of less than 0.05, and a fold change cut-off v alue of 1.5. Among the 481dysregulated metabolites, 274 metabolites were up-regulated, while 207 metabolites were down-regulated in the DOCK8 deficiency group. However, only 40 metabolites were positively identified using the dansyl standard library based on exact mass and retention time match for the metabolite and its labeled internal standard. (

Table A1.10**)**

Similarly, the binary comparison between AD patients and Ctrl groups (**Error! Reference s ource not found.D)**, showed clear cluster separation between the two groups (Q2=0.962) and a total, of 418 metabolites were dysregulated including 232 up-regulated and 186 down-regulated metabolites **(Error! Reference source not found.E)**. In this group, only 37 metabolites w ere positively identified using the dansyl standard library (Error! Reference source not found.)

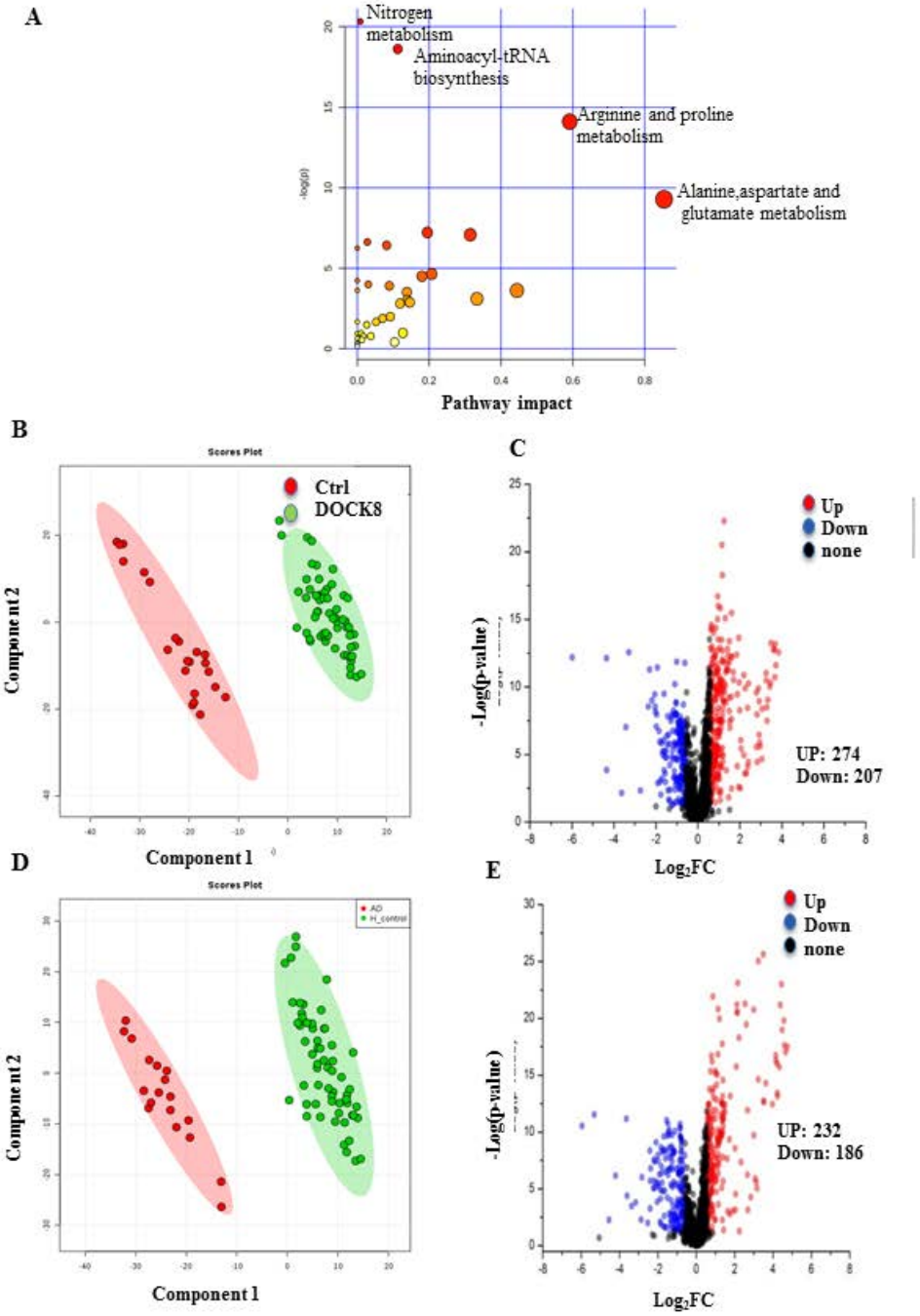


Figure 0.3: Pathway analysis and binary comparisons (serum). (**A**) Pathway analysis for DOCK8 deficient patients vs Ctrl comparison**. (B)** DOCK8 deficient patients (n=10 in duplicates) vs Ctrl (n=33 in duplicates): PLS-DA score plot with a calculated space Q2=0.971 and R2=0.997. **(C)** Volcano plots (DD vs. ctrl) with fold change > 1.5 (up-regulated=274 metabolites) and < 0.67 (down-regulated=207 metabolites); q=0.049, p=0.107, 40 metabolites were positively identified. **(D)** Atopic dermatitis (AD) (n=9) vs Ctrls (n=33): PLS-DA score plot, with a calculated space Q2=0.962 and R2=0.998**. (E)** Volcano plots with fold change > 1.5 (up-regulated=232) and < 0.67 (down-regulated=186), q=0.050, p=0.055, total 37 metabolites were positively identified. Abbreviations: DOCK8-Dedicator of cytokinesis 8, AD-Atopic dermatitis, PLS-DA-Partial least square discrimination analysis.

Seven metabolites were positively identified using the dansyl standard library after a binary comparison between DOCK8 deficiency and AD cohorts **(Figure 0.4A, B)** while, a total of 147 metabolites were dysregulated (118 and 29 metabolites were up and downregulated in DOCK8 deficiency group, respectively). The seven positively identified metabolites are presented in **Figure 0.4C-I.** (**Table A1.12)** Among those, aspartic acid and 3-hydroxyanthranillic acid were significantly upregulated in DOCK8 deficiency patients, whereas the dipeptides leucyl-phenylalanine and glycyl-phenylalanine were downregulated compared to the AD. Hypotaurine, guanosine, and 2-aminooctanoic acid were not found to be significantly differentially expressed in DOCK8 deficiency compared to AD after using one way ANOVA / post-Tukey's method **(Figure 0.4 G-I).**

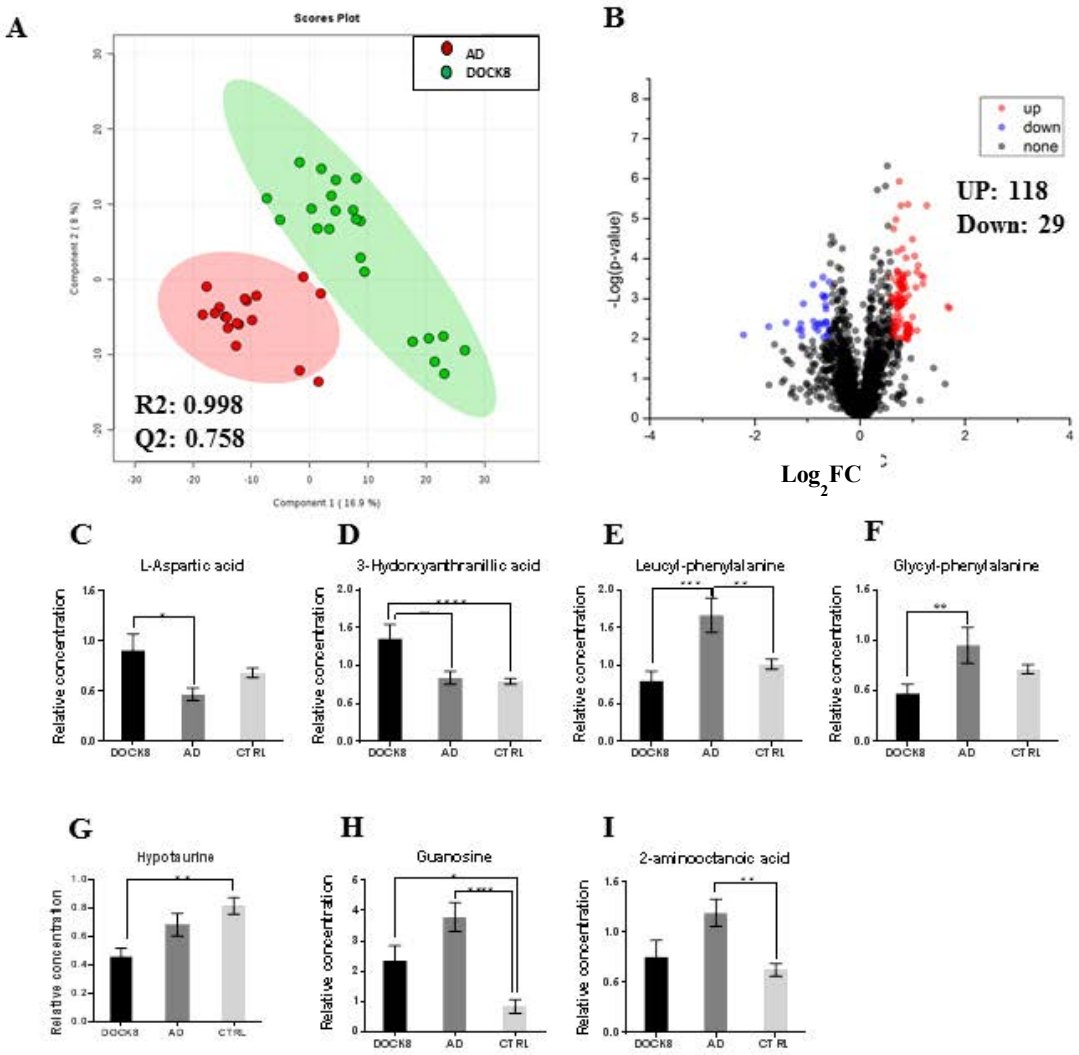


Figure 0.4: **Positively identified serum metabolites in DOCK8-deficient patients vs AD vs Ctrls**. (A) PLS-DA score plot for binary comparison between DOCK8 deficient and AD, with a calculated space Q2=0.758 and R2=0.998. (B) Volcano plot with fold change > 1.5 (up-regulated=118) and < 0.67 (down-regulated=29), total 7 metabolites were positively identified. (C) L-Aspartic acid is up-regulated in DOCK8 deficient compared to AD patients. (D) 3-Hydroxyxanthranillic acid is up-regulated in DOCK8 deficient patients. Dipeptides leucyl-phenylalanine and glycyl-phenylalanine are up-regulated in AD patients compared to DOCK8 patients (E, F respectively). (G) Hypotaurine is down-regulated in DOCK8 deficient patients compared to Ctrl.(H) Guanosine is up-regulated in DOCK8 deficient and AD patients while (I) 2-aminooctanoic acid is up-regulated in AD patients only. For paired analysis, a combination of t-test and fold change analyses is represented in this volcano plot, where the x-axis (FDR-corrected p.value), and y-axis is true positive. Statistical analysis was performed using one way ANOVA, post hoc Tukey's, where * Indicates significance with p-value <0.05, ** p-value <0.001, and otherwise not significant (ns). Abbreviations: DOCK8-Dedicator of cytokines8, AD-Atopic dermatitis, Ctrl-healthy controls. PLS-DA-Partial least square discrimination analysis

4.4.3 Biomarker Evaluation

As a result of the binary comparisons between DOCK8 deficiency vs Ctrl, AD vs Ctrl, and DOCK8 deficiency vs AD groups, receiver operating characteristics (ROC) exploring curves were generated **(Error! Reference source not found.A).** The 95% confidence interval was c alculated for these curves using 500 bootstrappings, and the optimal cutoff was determined using the furthest to diagonal line (Youden) to evaluate the sensitivity and specificity of the potential metabolites for being differentiating potential biomarkers mainly between DOCK8 deficiency and AD patients. The combination of the top metabolites in ROC curves, show AUCs ranging from 0.85-0.93**(Error! Reference source not found.A).** The significant features o f the positively identified metabolites **(Error! Reference source not found.B)** shows aspartic acid and 3-hydroxyanthranillic acid are being up-regulated in DOCK8 compared to AD, whereas hypotaurine is down-regulated compared to ctrl. Leucyl-phenylalanine and glycyl-phenylalanine, were down-regulated in DOCK8, compared to AD, guanosine is upregulated in DOCK8 when compared to ctrl and 2- aminooctanoic acid is up-regulated in AD compared to ctrl.The combination of all seven analytes gave the maximum confidence of differentiation and detection of DOCK8 deficiency from the AD with (AUC=0.922). ROCs for Hypotaurine, 3-hydorxyanthranillic and glycyl-phenylalanine are represented in **Error! Reference source not f ound.C-F** respectively.

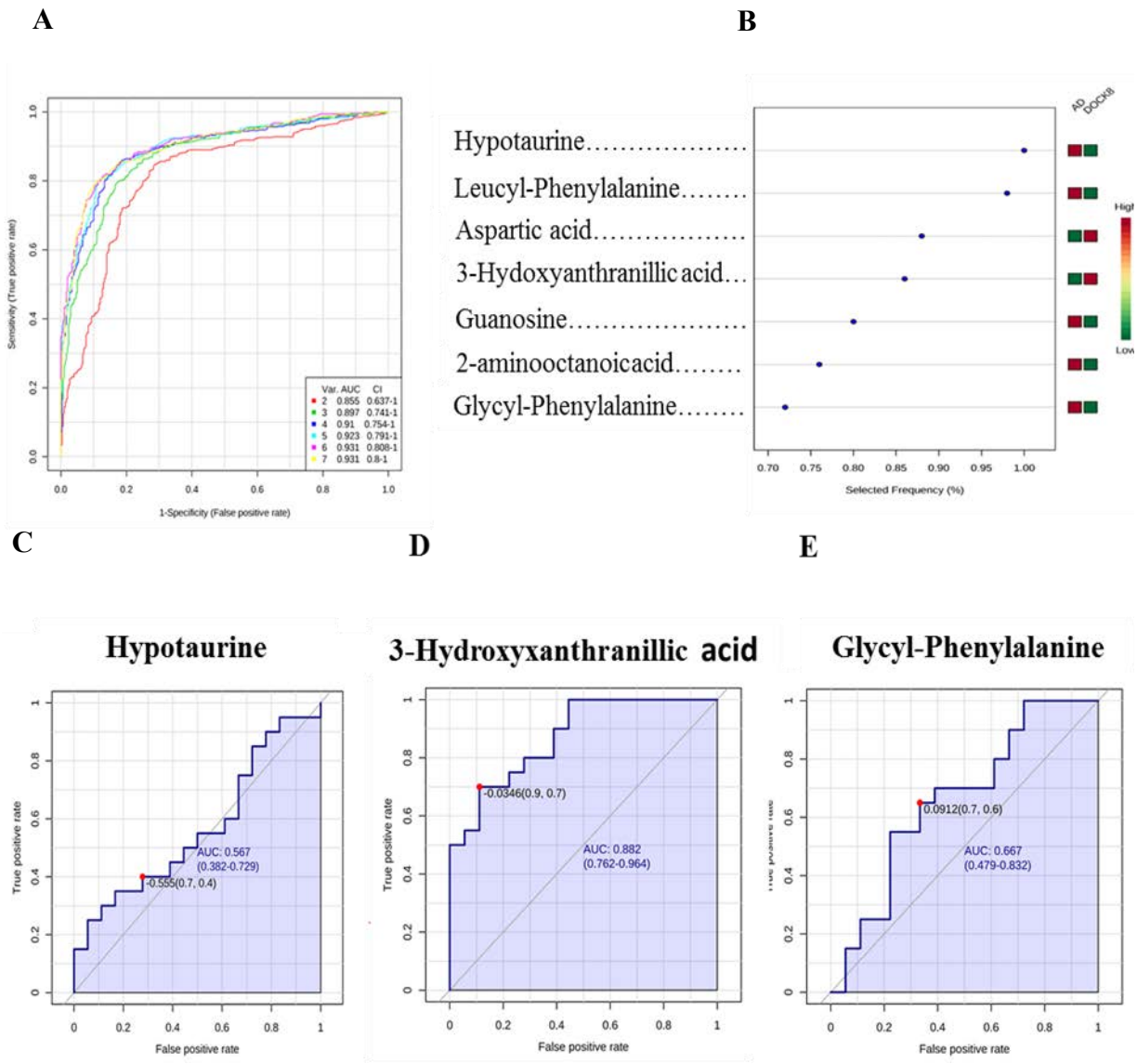


Figure 0.5: Receiver operating characteristics (ROC) curve and loading plots for positively identified serum metabolites in the comparison between DOCK8-deficient vs AD patients. (A) ROC generated by random forest model shows area under the curve (AUC)=0.931 for identified metabolites. (B) Loading plots for the 7 positively identified metabolites shows upregulation of aspartic and 3-hydroxyanthranilic acids in DOCK8 deficient patients. (C) Hypotaurine is not significantly differentially expressed in DOCK8 compared to AD patients, AUC- 0.567 and p value of 0.41537. (D) 3-Hydroxyxanthranillic acid is significantly upregulated in DOCK8 patients, AUC: 0.882 and p value of 4.4491E5. (E) Glycyl-phenylalanine is downregulated in DOCK8 deficient patients compared to AD patients, AUC: 0.667 and p value of 0.04766. Data was normalized, transformed, scaled by median, log, and Pareto scaling to make sure all the data are under Gaussian distribution. For paired analysis, a combination of t-test and fold change analyses is represented, where the X-axis (FDR-corrected p. value), and Y-axis is true positive.Abbreviations: DOCK8-Dedicator of cytokines8, AD-Atopic dermatitis, Ctrl-healthy controls.

The PBMC cell line metabolomics profiles were generated using the same mass spectrometry platform. Metabolomics profiles were generated in cell lystates for DOCK8 deficiency, AD, and Ctrl groups which showed cluster separation among them **(Figure 0.6)** Pathway analysis revealed multiple perturbations. PLS-DA analysis showed cluster separation in the binary comparisons: DOCK8 (n=7) vs Ctrl (n=4), AD (n=4) vs Ctrl (n=4), DOCK8 deficiency vs AD.(**Figure 0.6C)** 4-Hydroxybenzoic and 3-hydroxymandelic acids were the common differentially expressed metabolites in both the PMBC and serum samples of DOCK8 deficient patients when compared with controls. Whereas when AD cohort samples were compared to ctrls, glutamic acid, ornithine, serotonin, 1, 4-diaminobutane and aniline (a primary aromatic amine) were in common among the serum and cell line samples. No common metabolites were observed among the serum and cell lines samples for the binary comparison between DOCK8 deficiency and AD patients.

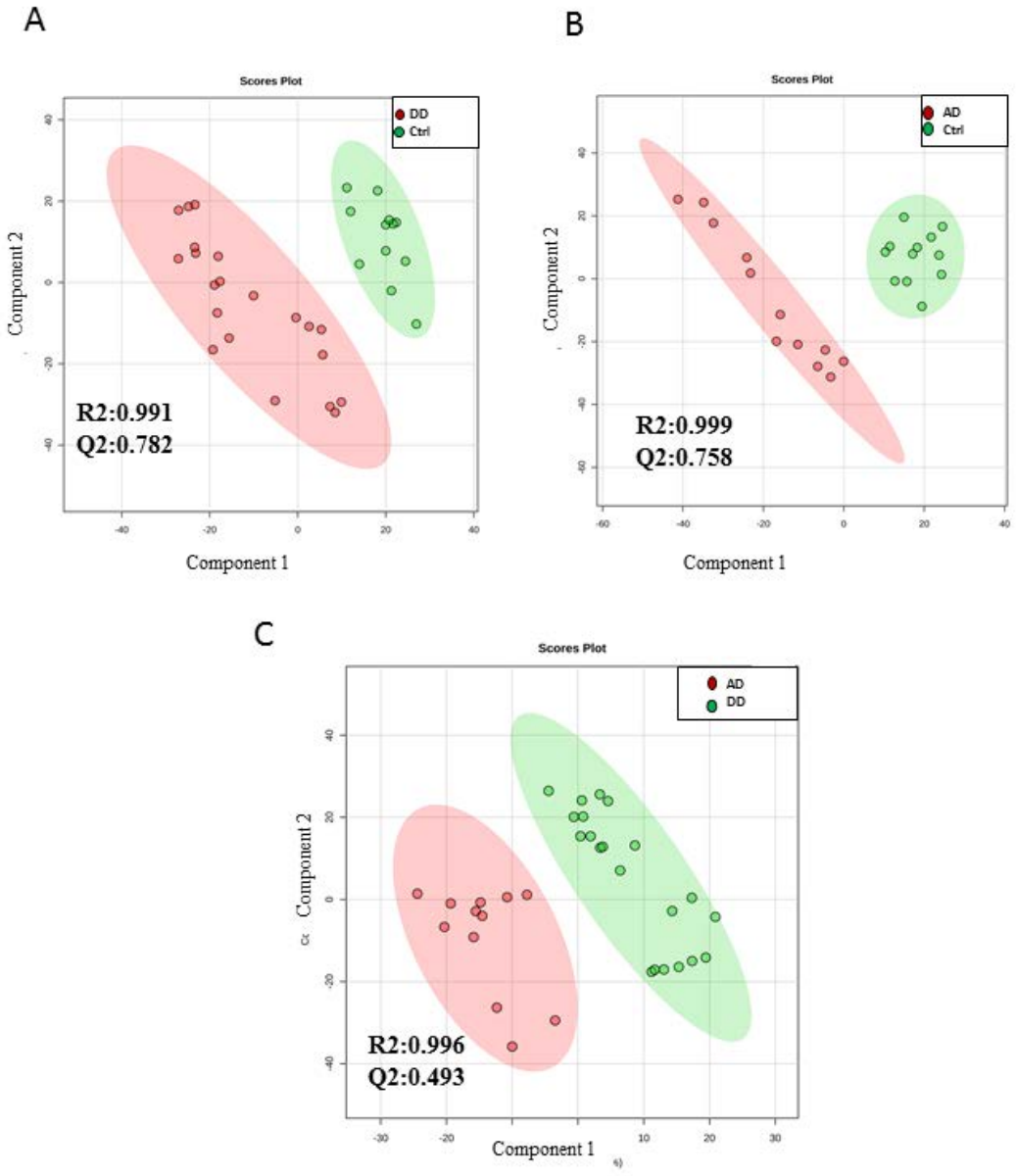


Figure 0.6: Binary comparisons of cell lines lysates metabolomic profiles (run in triplicates) show distinct patterns in DOCK8 deficient vs. atopic dermatitis patients. (**A)** PLS-DA score plot in DOCK8 deficient patients (DD) (n=7) vs Ctrl (n=4) with a calculated space Q2=0.782 and R2=0.991. **(B**) PLS-DA score plot in AD (n=4) vs Ctrl. (n=4) with a calculated space Q2=0.758 and R2=0.999. (**C)** PLS-DA score plot for binary comparison between DD and AD, with a calculated space Q2=0.493 and R2=0.996.(Abbreviations: DOCK8-Dedicator of cytokinesis 8, AD-Atopic dermatitis, Ctrl-healthy ctrls, PLS-DA-Partial least square discrimination analysis.)

4.5 Discussion

It is critical to recognize DOCK8 deficiency and differentiate its various clinical and molecular forms before severe life-threatening complications arise. For example, Aydin, et.al (2015) in a study on 136 DOCK8-deficient patients, reported malignancies in 17%, life-threating infections in 58% and non-infections cerebral events in 10% of their patients.(23)Differentiating DOCK8-deficient from AD patients can be difficult in infants and young children because of overlapping clinical and laboratory findings. The DOCK8 protein regulates intracellular signaling networks, proliferation, differentiation, migration, synapsis formation, adhesion and survival of cells affecting innate and adaptive immunity reflecting complex function.(3, 23, 24)

The identification of predictive biomarkers to distinguish DOCK8-deficient from AD, based on serum metabolite changes, requires a highly sensitive platform to allow the detection of very low abundant (pmol to fmol) metabolites. Chemical isotope labeling LC-MS represents a robust method for metabolomics profiling and biomarker discovery, as the ^{13}C- labeled pool served as an internal standard and compensated for the fluctuations in MS response.(25) In this study, seven metabolic features were found to significantly differentiate between DOCK8-deficient and AD patients. Taken together, these seven differentially expressed metabolites paint a distinctive metabolomics profile in DOCK8-deficient and AD **(Figure 0.4C-I**). Upregulation of 3-hydroxyanthranilic acid was observed in DOCK8-deficient cohort compared to Ctrl and AD, while aspartic acid was upregulated in DOCK8-deficient cohort compared to Ctrl and hypotaurine was down-regulated in DOCK8 deficiency compared to the AD.

The binary analyses between DOCK8 deficiency with and without various clinical complications (asthma, bronchiectasis, molluscum contagiosum, sclerosing cholangitis,

candidiasis, warts, sinusitis, or malignancy) failed to demonstrate a secondary role for these phenotypes on the overall DOCK8 deficiency specific metabolites (**Figure 0.7)**, which suggests that these metabolites are primarily due to the underlying genetic deficiency, rather than its secondary medical complications.

The hypotaurine-taurine metabolism pathway starts with cystamine production via cystine decarboxylation, which is then reduced to cysteamine followed by oxidation to hypotaurine (by the enzyme cysteamine dioxygenase). Hypotaurine is ultimately oxidized to the final product taurine by hypotaurine dehydrogenase, which is then excreted out of the body or used within. While very little is known about the physiological role of hypotaurine, taurine is known to have diverse cellular functions including neurotransmission, retinal photoreceptor differentiation through taurine-upregulated gene 1 (TUG1), a non-coding RNA that modulates the expression of photoreceptor-specific genes in the retina (27) , osmoregulation, calcium modulation and suppression of inflammation, as well as normal mitochondrial respiratory chain function.(28) Within the CNS, taurine exhibits an age dependent gradient expression and has its own synthesizing enzymes, receptors and transporters.(29) Taurine has also been implicated as a tumor marker in a number of different cancer types. (30-33) The exact mechanisms regulating taurine levels in tumors have not been established but may involve either regulation of its synthesis from hypotaurine and/or regulation of its uptake from the extracellular environment, mediated by the taurine transporter SLC6A6. Mammals oxidize hypotaurine to taurine using trace amounts of hydrogen peroxide (H_2O_2) produced by cellular metabolism, which is likely to be more frequent in the brain than the liver.(26) Holopainen et al.(1982) demonstrated the rapid uptake of hypotaurine into neuroblastoma cells suggesting that hypotaurine may have a function in the regulation of neuronal activity.(27) Other studies suggested a role for hypotaurine as an antioxidant and protective

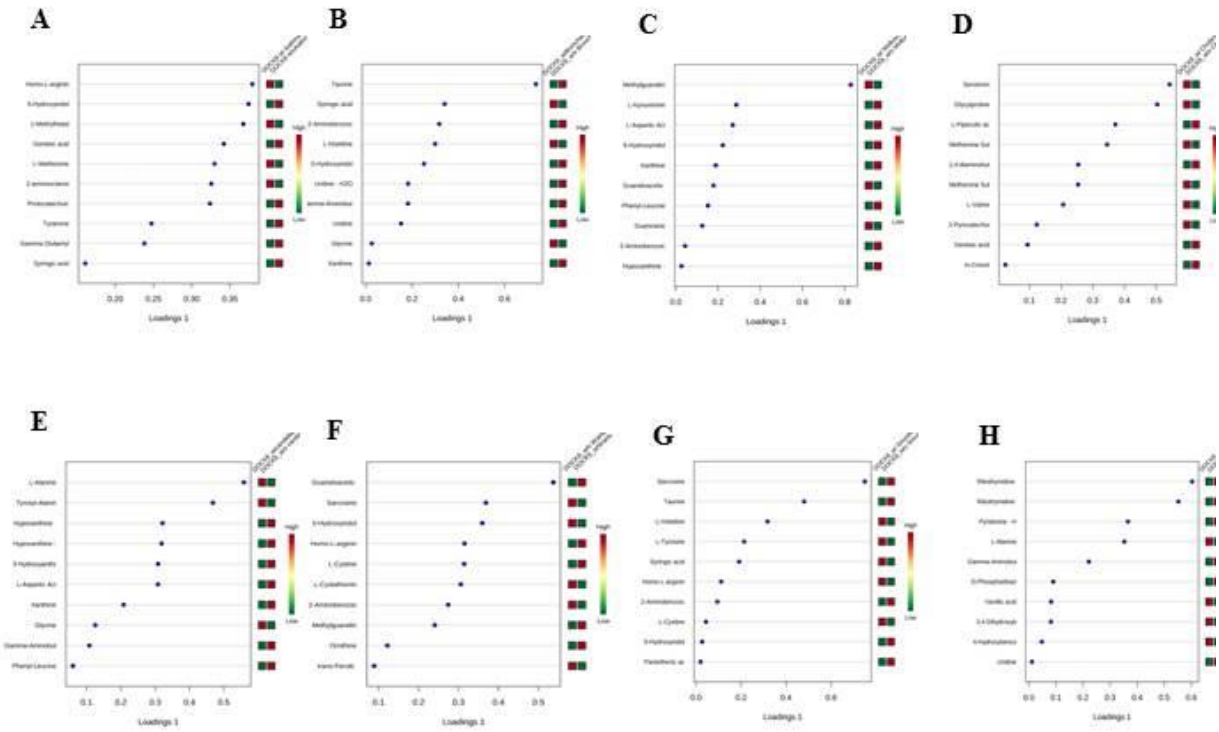


Figure 0.7 : Impact of DOCK8 phenotypes on metabolomic profiling. PLS-DA loading plots based on binary comparisons in DOCK8 deficient patients with (+) and without (-) various clinical phenotypes including (**A**) Asthma, (**B**) Bronchiectasis, (**C**) Molluscum contagiosum, (**D**) Sclerosing cholangitis, (**E**) Candidiasis, (**F**) Warts, (**G**) Sinusitis, (**H**) Malignancy

agent under physiological conditions.(28, 29) Peng et al. (2016) also showed that under hypoxic signaling, hypotaurine behaves as an oncometabolite promoting tumor progression.(30) The observation of underexpression of hypotaurine in the DOCK8 deficient patients suggests potential loss of its antioxidant and protective effects.

3-Hydroxyanthranillic acid (3-HAA), a tryptophan catabolism molecule produced through the kynurenine pathway, suppresses antitumor immunity in human malignancy, (31) has immune regulatory properties as it can inhibit Th1 and Th2 cells, increase the percentage of regulatory T-cells, and regulate leucocyte infiltration and plaque formation.(32) It is found in the human epidermis where it participates in multiple enzymatic reactions.(33, 34) In addition, 3-HAA appears to play an essential role in the pathogenesis of several inflammatory, infectious and degenerative diseases.(35) The increased tryptophan catabolism, in relation to infections during the course of the disease may lead to the increased levels of 3HAA as seen in our DOCK8 patients (Figure 3B). More recently, Hongjun et al.(2017) showed that homozygous germline deficiency in 3-hydroxyanthranilic acid 3,4-dioxygenase (HAAO) causes niacin deficiency, responsible for a complex human multiple congenital (cardiac, vertebral, renal) anomalies syndrome, which can be averted in affected mice via prenatal niacin supplementation.(36) Moreover, 3-HAA decreases Th1 and Th2 responses (37) and directly impair immune responses via different mechanism like the ability to induce the expression of hemeoxyhenase-1 (HMOX-1), (35) cell cycle control and inhibition of cytokine release through the depletion of intracellular gluthathione, (38) as well as the induction of antigen specific IL-10 producing T cells (32) and inhibiting PKD1, which is essential mediator of CD28 in inflammation.(39) Dietary aspartic acid down-regulates TLR 4, Nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and Myeloid differentiation primary response 88 (MYD88) in the jejunum mediate the inflammatory response, thereby affecting the growth performance and inflammation in piglets.(40) Furthermore, intraperitoneal

injection of aspartic acid in mice has been found to have more beneficial effects on Experimental autoimmune encephalomyelitis, (EAE) a widely used model for multiple sclerosis, reflecting upon the role of aspartate in therapy.(41)

Perturbations in amino acid metabolism had been also observed in some cancers as well as neurodegenerative disorders such as Alzheimer disease and Parkinson diseases.(40-42) Aspartic acid is a major excitatory neurotransmitter, which had been found to be increased in some epileptic and stroke patients, decreased in patients with depression and brain atrophy. In contrast, guanosine is a nucleoside that exerts important neuroprotective and neuromodulatory roles in the central nervous system, which may be related to inhibition of the glutamatergic neurotransmission activity. Glycyl-phenylalanine, a dipeptide produced by incomplete protein catabolism, consists of glycine and phenylalanine and is known to play an essential role in cell signaling effects by impacting specific amino acid degradation pathways.(43) It is transported intact by a cation independent facilitative diffusion mechanism during which the dipeptide is hydrolyzed to its component amino acids.(43) Some dipeptides have physiological or cell-signaling effects although most are simply short-lived intermediates on their way to specific amino acid degradation pathways following further proteolysis. This dipeptide has not been identified yet, in human tissues or biofluids and so it is classified as an 'expected' metabolite. 2-aminooactanoic acid is shown to be perturbed in human colorectal cancers.(44) Taken together, these findings call for further analysis of the perturbed amino acid pathways for additional insight into its significance.

In summary, DOCK8 deficiency appears to be associated with a distinctive metabolomics profile characterized by significant differential overexpression of 3-HAA and aspartic acid both of which have been linked to oncogenesis coupled with underexpression of hypotaurine, guanosine, and the dipeptides leucyl-phenylalanine, glycyl-phenylalanine, which together seem to contribute to some of the immune and malignancy-related phenotypes observed in

this disease. The complex nature of these diseases suggests that no single biomarker will be sufficient to meet the clinical needs of such patients; instead, a larger panel of biomarkers will ultimately be required. These findings may inform further mechanistic analyses in these diseases. In the next chapter, Metabolomics and cytokines profiling of food allergy was studied in DOCK8-deficient and atopic dermatitis patients as both the cohort groups have food allergies in common. ISAC sensitization profiling showed differential and overlapping sensitization patterns.

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4.7 Chapter 4 summary

- Metabolomic profiling differentiated DOCK8 deficiency from atopic dermatitis and revealed distinct biochemical pathway perturbations .The aim of this study was to find metabolomics profiles distinguishing the study groups. CIL LC-MS targeting the amine/phenol sub-metabolomes was used to find novel differentially expressed biomarkers in DOCK8 and AD patient groups.
- Seven metabolites were positively identified in binary comparisons between DOCK8-deficient and AD patients. Aspartic and 3-hydroxyanthranillic acids were upregulated in DOCK8 deficiency, whereas hypotaurine, leucyl-phenylalanine, glycyl-phenylalanine and guanosine were downregulated.
- Hypotaurine, 3-hydroxyanthranillic acid and glycyl-phenyalanine were identified as potential biomarkers specific for DOCK8 deficiency
- The complex nature of these diseases suggests that no single biomarker will be sufficient to meet the clinical needs of such patients; instead a larger panel of biomarkers will ultimately be required. Further validation of these biomarkers in larger cohorts is necessary for both discrimination and establishing prognosis among these patients.

CHAPTER 5: DISTINCT IgE BASED ALLERGEN ARRAY, METABOLOMICS AND CYTOKINE PROFILES IN DOCK8-DEFICIENT AND ATOPIC DERMATITIS PATIENTS

5.1 Introduction

Elevated serum IgE and eczema are indicators for most allergic diseases. However, this cannot be considered as very specific markers as these are also elevated in patients suffering from primary immunodeficiency disorders (PID), such as the Hyper IgE syndromes (HIES). Loss of function mutations in the dedicator of cytokine8 (DOCK8) gene causes the autosomal recessive HIES and are characterized by highly elevated serum IgE levels, recurrent staphylococcus skin abscesses and mucocutaneous infections, severe eczema and pulmonary infections. Furthermore, these patients suffer from autoimmunity and increased susceptibility to virus-induced malignancies and lymphomas.(1)Due to the high morbidity and mortality associated with DOCK8 deficiency, hematopoietic stem cell transplantation (HSCT) is the preferred definitive therapy for this disorder.(2)

As discussed in chapters 2 and 4, atopic dermatitis (AD) is a chronic inflammatory disease with remission that commonly occurs in early infancy. Clinical manifestations in patients with AD include food allergy, asthma, and allergic rhinitis, probably in response to impaired innate and adaptive immunity as well as environmental stimuli.(3) Eczematous skin lesions, pruritus and food allergies are common overlapping clinical features in DOCK8-deficient and AD. Food allergy is a typical type 1 hypersensitivity reaction, occurring when patients develop specific IgE antibodies because of exposure to some food proteins via the skin, gut or respiratory system. In general, an active oral immune tolerance is generated against food and allergic reactions in humans are caused by a disruptive adaptive immune system that generates a specific immune response upon exposure to certain foods.(4)

The immuno Solid-Phase Allergen Chip (ISAC) (Thermo Fisher, Uppsala, Sweden) microarray technique is preferred over routine allergen specific IgE testing using the ImmunoCAP, because the ISAC panel includes purified allergen components from 51 allergenic sources allowing the determination of specific IgE against 112 allergen

components in the same serum sample. As detailed in Chapter 1, metabolomics, refers to a comprehensive and systematic characterization and quantification of endogenous and exogenous small molecules with molecular size <1500 Da (metabolites), using high throughput technologies. Interestingly, only few reports have documented the use of metabolomics in food allergy. Kong et.al (2015) used liquid chromatography MS analysis and showed that elevated levels of uric acid played a critical role in the induction of peanut allergy. The authors hypothesized that uric acid activates dendritic cells by increasing CD86 and OX40 ligand expression.(5) To date, no data is available on the relationship between the IgE-mediated food sensitization in DOCK8 deficient and AD patients and the metabolomic expression.

In this study chemical isotope labeling liquid chromatography-mass spectrometry (CIL LC-MS) was used to understand the metabolic fingerprinting in food allergy in the study cohort, which may help to understand the inflammatory responses that develop during DOCK8 pathogenesis. Since DOCK8-deficient and AD patients have detectable environmental, food and microbial antigen-specific IgE titers, the comprehensive evaluation of food and aerosolized allergens sensitization patterns in patients with DOCK8-deficient and AD patients was associated with perturbations in metabolites and cytokines expression.

5.2 Aims

The aims of this study were as follows:

1) To evaluate food and microbial antigen-specific IgE titers in DOCK8-deficient and AD cohorts, using ImmunoCAP Solid-Phase Allergen Chip (ISAC).

2) To analyse and understand the metabolic fingerprinting in food allergy using chemical isotope labeling liquid chromatography-mass spectrometry (CIL LC-MS)

3) To analyse the cytokine profiles in these patients and correlate them with the metabolic perturbations

5.3 Methods

5.3.1 Serum samples

As discussed in chapter 2 and 4, the study cohort for DOCK8 and AD patients were same. (6, 7)This study was approved by the Research Ethics Committee at the Office of Research Affairs, of King Faisal Specialist Hospital and Research Center (KFSHRC) (RAC No. 2160 015).

5.3.2 Allergy Microarray (ISAC)

Serum samples from DOCK8-deficient (n=10), AD (n=9) and control samples (n=3) underwent testing for ISAC. (Thermo Fisher, Uppsala, Sweden) ISAC is a multiple allergen component assay based on a solid phase multiple immunoassays, containing immobilized protein, which detects and quantifies IgE antibodies to 112 allergen components. The ImmunoCAP ISAC IgE chip was placed in the humidity chamber with the reaction sites facing upwards, 20µl of each sample along with IgE control serum was aliquoted onto one reaction site and incubated at room temperature for 120 minutes. The chip was washed with 220 ml of buffer for 10 minutes followed by a washing step with 220ml of purified water for 5 minutes and the chip was allowed air dry. 20 µl of IgE detection antibody solution was added onto each reaction site of the chip, and incubated at room temperature for 60 minutes. The non-binding detection antibody was removed by rinsing gently under distilled water was removed by rinsing gently under distilled water. Finally, the chip was again washed with 220 ml of buffer A and purified water and allowed to air dry. The serum IgE antibodies bind to the immobilized allergen components and in turn the allergen-bound specific-IgE antibodies are detected by fluorescence signal, measured with a confocal laser scanner, and the microarray image data analyzed using ImmunoCAP ISAC Xplain software (Phadia).(6) The list of 112 allergens (food and aero-allergens) is listed in

Table A1.13. Results are reported in ISAC standardized units (ISU) and categorized based on manufacturers cutoff levels (0.3 to 0.9= low, 1 to 14.9=moderate, >15=very high).

5.3.3 Chemical Isotope Labeling Liquid Chromatography-Mass Spectrometry

As discussed in chapter 4, CIL LC-MS metabolomics approach was used, serum samples for DOCK8 and AD cohorts were analyzed using a Thermo Fisher Scientific Dionex Ultimate 3000 UHPLC System (Sunnyvale, CA) linked to a Bruker Maxis II quadrupole time-of-flight (Q-TOF) mass spectrometer (Bruker, Billerica, MA).(7)

Cytokines/Chemokine profiling

Patient samples were quantitatively profiled using the MILLIPLEX MAP kit, (Millipore) phycoerythrin-conjugated detection was used to form sandwich complexes, and the fluorescence data acquired on the FLEXMAP 3D as described in chapter 2.(6)

Data and statistical analysis

Metabolomics data were aligned based on peak's accurate mass and retention time; the metabolites were positively identified by putative identification against DnsID Library (www.mycompoundid.org). Putative identification was performed by searching accurate mass against My Compound ID library, which contains 8,021 known human metabolites and 375,809 predicted metabolites.(9, 10) Univariate analysis (volcano plot) for significant metabolites was performed based on a criterion of fold-change of greater than 1.5 or less than 0.67 with q-value (false discovery rate) less than 0.05. The sparse partial least squares discriminant analysis (sPLS-DA) were performed using MetaboAnalyst (http://www.metaboanalyst.ca). The raw data were normalized to the sample total median to ensure all samples were normally distributed. To visualize the difference among the study groups and make individual features more comparable, data were log-transformed, and Pareto scaled respectively. Binary comparison between allergen positive and negative groups in

DOCK8-deficient and AD patients were performed to find the significant metabolites followed by pathway analysis.

Two-tier ID approach was used to perform metabolite identification. In tier 1, peak pairs were searched against a labeled metabolite library (CIL Library) based on accurate mass and retention time. The CIL Library (amine/phenol channel) contains 711 experimental entries, including metabolites and dipeptides. 190 peak pairs were positively identified in tier 1. In tier 2, linked identity library (LI Library) was used for identification of the remaining peak pairs. LI Library includes over 2000 human endogenous metabolites from 68 metabolic pathways, providing high-confidence putative identification results based on accurate mass and predicted retention time matches. 97 peak pairs were putatively identified in tier 2. The targeted statistical analysis of multiple study groups were carried out using Graph Pad Prism (version 6.0, Graph Pad software). As the data were Gaussian distributed, unpaired two-tailed Student's t-test was used for binary comparison between any two study groups. In all experiments, a calculated p-value<0.05 was considered to be statistically significant.

5.4 Results

DOCK8- deficient and AD patients were recruited from the Allergy and Immunology clinic at KFSHRC, generally presented with increased serum IgE levels, eczema, and eosinophilia, as described earlier.(6) In DOCK8- deficient cohort, 50% were females and presented with higher median IgE levels (14355 KU/L) compared to AD patients (1387 KU/L). IgG and IgA median score was also higher in the DOCK8- deficient compared to AD cohort, 18.35g/L±3 and 3.1g/L±1.3, respectively, whereas IgM median score was lower in DOCK8- deficient cohort (0.35 g/L), compared to AD (0.92g/L). Median scores for monocytes were found to be higher in DOCK8- deficient patients (9.5%10^9/) compared to AD (7.5%10^9/), in contrast

to lymphocytes and basophils which showed a lower median score in DOCK8- deficient cohort of17 x10^9/L and 0.55x10^9/L, respectively. However, eosinophils showed a higher median score in DOCK8- deficient group (28.9%) compared to the AD. (13%) **(Error! R eference source not found.)**

5.4.1 Clinical manifestation

Eczema, dry skin and food allergies were common to both groups of patients **(Error! Reference s ource not found.)**.Staphylococcus infections and pneumonia were the most common clinical features seen in the DOCK8-deficient patients, whereas rhinitis was only seen in the AD group. Seven of ten DOCK8-deficient patients, presented with asthma whereas only four of nine AD patients had asthma. DOCK8-deficient patients also presented with viral infections, warts, otitis media sinusitis, seizures, and cataract, as described in chapter 2.(6) The Severity Scoring of Atopic Dermatitis (SCORAD) and the Visual Analogue Scale (VAS), were comparable for both DOCK8-deficient and AD groups. (11) CXCL10, TNF-A, EGF cytokines were also calculated as they are typified signature biomarkers to distinguish DOCK8-deficient compared to AD. (6) The median value for CXCL10 and TNF-A were found to be higher in DOCK8-deficient patient, whereas in AD patients EGF levels were higher compared to DOCK8 patients. **(Error! Reference source not found.)**

5.4.2 ImmunoCAP data

ImmunoCAP data was obtained from patient records for seven out of nine of the DOCK8-deficient patients. All DOCK8-deficient patients were allergic to milk, tree nut and wheat, followed by six patients who were allergic to egg and soybean. In contrast, among AD patients, eight patients were allergic to wheat, soybean and tree nut followed by seven patients allergic to milk and six patients allergic to egg. Only one patient from the DOCK8-

deficient and AD groups showed sensitization towards shellfish. There was no overall significance observed between the two cohorts in food-allergens or aero-allergen groups.

(**Figure 0.1) (**

Table 0.1)

Table 0.1: Quantitative IgE testing using ImmunoCAP

	DOCK8 (n=7)							AD (n=9)								
Patient name	**P1**	**P2**	**P3**	**P4**	**P8**	**P9**	**P10**	**P11**	**P12**	**P13**	**P14**	**P15**	**P16**	**P17**	**P18**	**P19**
Food allergens																
Egg white	1	6	3	0	1	3	5	5	2	0	2	1	0	3	0	3
Egg yolk	0	4	1	0	2	0	3	0	0	0	1	1	0	0	0	2
Milk	3	6	4	2	6	4	4	3	1	0	0	3	2	3	6	3
Wheat	2	5	3	1	6	5	4	6	3	1	1	2	0	2	4	3
Fish	0	0	0	0	1	1	0	3	0	0	0	1	0	0	2	2
Soyabean	3	5	3	1	4	5	0	6	2	1	3	2	0	3	6	3
Shellfish	0	1	0	0	0	0	0	0	4	0	0	0	0	0	0	0
Peanut	4	6	4	0	5	5	0	6	3	1	4	1	0	3	6	5
Brazilnut	3	0	0	0	6	0	0	0	1	0	4	1	2	3	0	3
Cashewnut	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	5
Walnut	0	0	0	0	0	0	0	0	0	0	0	4	0	4	0	6
Pistachio	0	0	0	0	0	0	0	0	0	0		3	0	5	0	6
Hazelnut	3	0	0	0	6	5	0	0	2	1	4	3	0	3	0	5

Class: <0.35=0; 0.35-0.70=1; 0.71-3.50=2; 3.50-17.50=3; 17.51-50.4=4; 50-100=5; >100=6

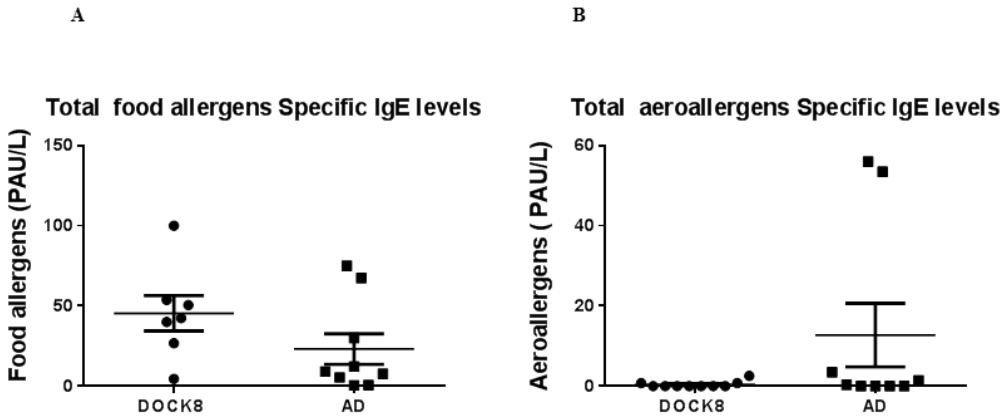

Figure 0.1: ImmnoCAP profiling in DOCK8-deficient(n=7) and atopic dermatitis(n=9) patients shows no significance.(A) Averaged concentration of food-allergens in PAU/L (B) Averaged concentration of aero-allergens in PAU/L, p-value< 0.05,Abbreviations: AD-Atopic dermatitis, PAU/L(Phadia arbitrary unit/L)

Table0.2 Clinical manifestations in DOCK8 deficient patients and AD patients

Diagnosis	Code	Age(yr)	Gender	IgE levels (KU/L)	IgM level (0.5-2.4 g/L)	IgG level (6-15.7 g/L)	IgA level (0.5-4 g/L)	RBC 10^ 12/L	WBC 10^9/L	CD4/CD8 Ratio	Lymphocytes 10^9/L	Monocytes 10^9/L	Basophils 10^9/L	Eosinophils 10^9/L
DOCK8	P1	21	F	25350	0.25	27.3	3.83	0.01	9.2	2.1	18.8	8.6	0.6	24.8
	P2	16	M	7530	NA	NA	NA	NA	NA	0.8	6	4	NA	35
	P3	16	F	1829	0.47	27.3	0.3	4.2	4.63	1	15.3	6.3	0.3	50.3
	P4	16	F	7250	0.25	13.6	2.54	5.9	4.9	0.73	21.4	10.5	0.2	11.4
	P5	5	M	16780	0.31	9.9	1.06	4.8	9.3	2.2	23	14.7	0.7	26.4
	P6	7	M	13160	0.45	15.4	5.6	5.29	9.93	0.7	18.7	13.5	0.5	11.7
	P7	14	M	15550	0.52	21.3	4.3	6.82	4.46	1.3	14	8.6	0.7	31.4
	P8	4	M	70.4	NA	NA	NA	5.3	24.9	9	8	1	1	34.8
	P9	9	F	21680	0.3	24.9	11.9	5.26	12.49	8.1	4	11		35.1
	P10	8	F	61060	0.25	4.5	0.5	3.81	21.42	3.2	34.4	16.5	0.4	12
Median		**11.5**		**14355**	**0.305**	**18.35**	**3.185**	**5.26**	**9.3**	**1.7**	**17**	**9.55**	**0.55**	**28.9**
AD	P11	21	M	6540	0.92	10	0.79	4.7	9.7	1.1	38.2	4.7	1	16.5
	P12	6	M	16500	0.62	20	2.63	5.2	10.6	0.9	33	7.7	0.5	15.6
	P13	15	M	1612	2.23	26	0.3	6.39	3.72	1.4	26.8	10.7	1.4	13.9
	P14	10	M	839	1.68	15	1.2	5.48	8.01	1.2	70.5	7	0.2	6.7
	P15	3	M	1221	0.88	16	0.98	5.3	6.59	1.3	57	5	4	13
	P16	8	M	659	1.64	15	1.3	5.22	5.88	1.2	57	5	4	13
	P17	13	F	1387	1.43	12	1.39	4.9	5.09	1.5	40.3	11.8	0.9	13
	P18	15	M	365	0.7	12.9	1.3	5.2	5.15	1.2	36.2	7.5	0.6	12.3
	P19	12	M	4587	0.7	8	0.3	4.71	9.63	1.4	53.2	9.3	0.5	7.9
Median		**12**		**1387**	**0.92**	**15**	**1.2**	**5.2**	**6.59**	**1.2**	**40.3**	**7.5**	**0.9**	**13**

* F-Female, M-Male, NA not applicable

5.4.3 IgE sensitization profiling

In order to have a standard allergic profile on all study patients, IgE sensitization profiling using ISAC revealed different sensitization patterns for food and aerosolized allergens. In our cohort, both aerosolized and food-allergens showed differences in IgE sensitization pattern within different food groups. The statistical analysis was performed on ISAC data by taking the sum of the respective allergens in each group (DOCK8-deficient, AD) and analysis of overall food and aerosolized allergen groups together showed differential expression pattern among the groups. The overall significance for both the groups is represented in **Figure A2 6.** Food allergen sensitisations were not different between groups, whereas aero-allergens were up- regulated in AD compared to the DOCK8-deficient cohort**.**

As represented in **Table A1.144**, among the food-allergens, DOCK8-deficient patients showed maximum sensitization towards cow milk (80%) and tree nut (70%), followed by egg (60%), soybean (60%), peanut (60%) and wheat (40%). In contrast, the DOCK8-deficient patients did not show any sensitization to shellfish, unlike the AD group (n=2). Within the food allergen group, the calculated highest median value for peanut allergens was observed in the AD cohort compared to DOCK8-deficient. A similar pattern was followed in egg and tree nut allergens, where egg allergen group showed higher IgE sensitization in the AD (P2, P7, and P9) compared to DOCK8-deficient patients (P18 and P11) (**Figure 0.2A-D).** Whereas for milk allergens, higher sensitization was seen in DOCK8-deficient patients, (P2, P8 and P9) compared to AD patients. (P11, P18 and P19) (**Figure 0.2D-H)** For overall peanut allergens (rAra h1, rAra h2, and rArah6), a slightly higher sensitization was observed in DOCK8-deficient (P7, P1, and P2) compared to AD patients. (P11 and P19) **Figure 0.3 (A-D)** Similar pattern were observed for soybean (**Figure 0.3 E-F)** and tree nut allergens.**Figure 0.4 (A-E)** In contrast, sensitization was slightly higher in AD patients for sesame (**Figure 0.3G)** (P11,

P12 P18 and P19) and for shellfish allergens (**Figure 0.5)** compared to DOCK8-deficient patients. No sensitization was observed for wheat and soyabean allergens. (**Figure 0.6**)

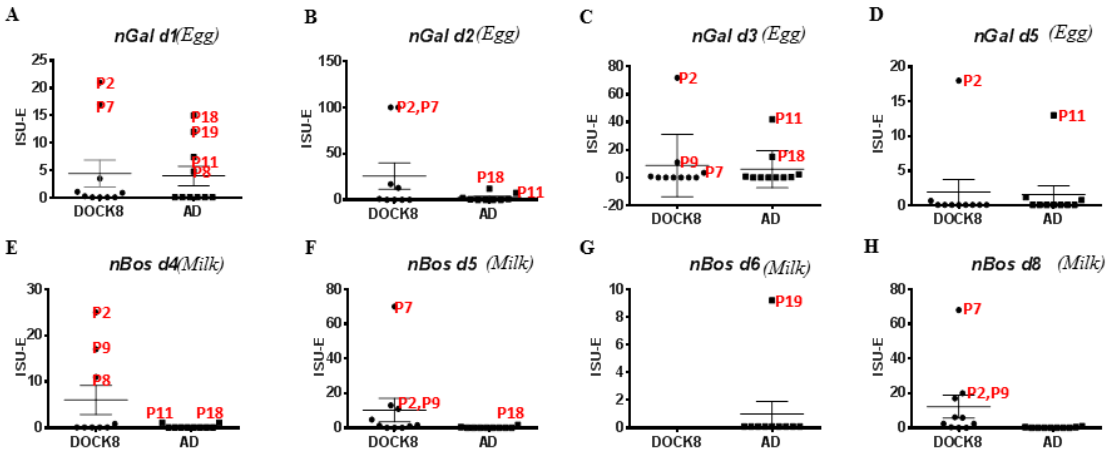

Figure 0.2: Egg (**A-D**) and milk **(E-H**) allergens sensitization pattern in DOCK8-deficient (n=10) and atopic dermatitis (n=9) patients using ISAC analysis. No stastical significance was observed in both the cohorts, P1-P10 are DOCK8-deficient, while P11-P19 are AD patients,p-value< 0.05, (Abbreviations: AD-Atopic dermatitis, ISAC-ImmunoCAP Solid-Phase Allergen Chip)

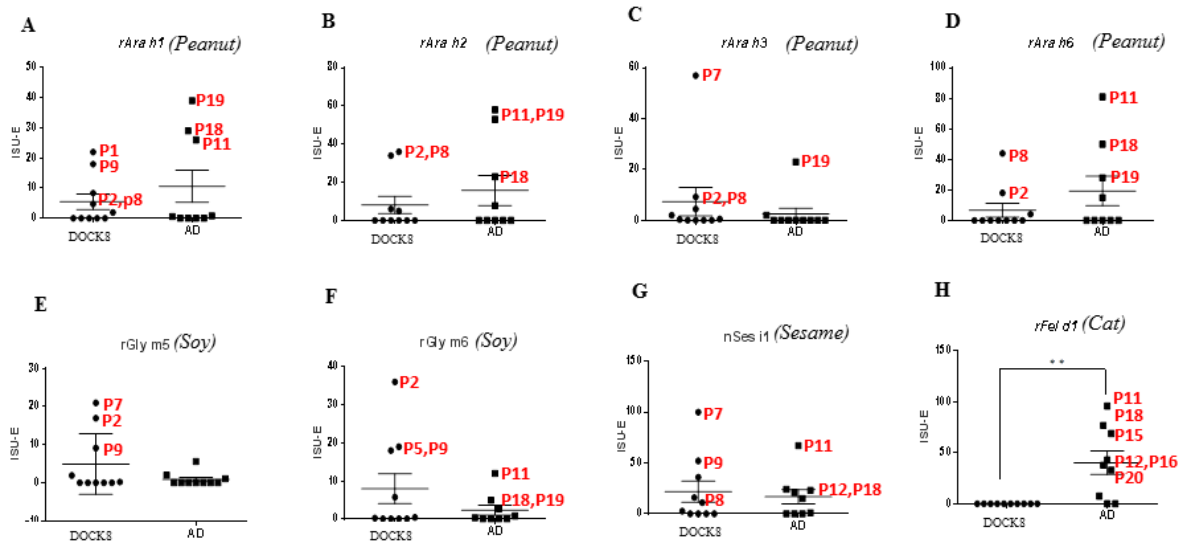

Figure 0.3: Food and aero-allergens sensitization pattern in DOCK8-deficient (n=10) and atopic dermatitis (n=9) patients using ISAC analysis: Cat allergen was up-regulated in AD in comparison to DOCK8 deficient patients. No significance was observed among the other allergens.* Indicates significance. Not specified ones are not significant) p-value< 0.05, P1-P10 are DOCK8-deficient, while P11-P19 are AD patients Abbreviations: (AD-Atopic dermatitis, ISAC-ImmunoCAP Solid-Phase Allergen Chip)

Table 0.3: Clinical profiles and key cytokine levels in DOCK8-deficient and AD patients

Diagnosis	Code	Eczema	Asthma	Rhinitis	Food allergy	Pnemonia	Staphylococcus infection	EGF (pg/ml)	CXCL10 (pg/ml)	TNFA (pg/ml)	VAS	SCORAD
DOCK8	P1	Yes	Yes	No	Yes	Yes	Yes	35.8	343.6	22.7	15.0	68.7
	P2	Yes	No	No	Yes	Yes	Yes	34.3	323.5	28.9	13.0	67.0
	P3	Yes	Yes	No	Yes	Yes	Yes	42.3	739.0	27.3	14.0	59.8
	P4	Yes	Yes	No	Yes	Yes	Yes	0.0	584.0	23.9	15.0	77.4
	P5	Yes	Yes	No	Yes	Yes	Yes	142.8	295.1	32.5	17.0	79.5
	P6	Yes	Yes	No	Yes	Yes	Yes	450.9	3724.0	32.1	15.0	62.5
	P7	Yes	NA	No	Yes	Yes	Yes	182.6	1229.0	23.7	17.0	70.6
	P8	Yes	Yes	No	Yes	Yes	Yes	225.7	2157.0	39.0	9.0	58.2
	P9	Yes	Yes	No	Yes	Yes	Yes	102.4	5127.0	107.9	14.0	58.8
	P10	Yes	No	No	Yes	Yes	Yes	64.2	994.3	20.8	12.0	70.1
Median								**83.3**	**866.7**	**28.1**	**14.5**	**67.9**
AD	P11	Yes	NA	NA	Yes	No	Yes	27.9	163.5	9.3	16.0	64.4
	P12	Yes	NA	NA	Yes	No	No	102.7	251.4	6.8	14.0	71.3
	P13	Yes	NA	NA	Yes	No	No	123.2	108.1	28.6	16.0	73.3
	P14	Yes	NA	NA	Yes	No	No	134.9	92.6	14.0	13.0	56.1
	P15	Yes	NA	NA	Yes	No	No	10.1	204.1	16.5	9.0	40.8
	P16	Yes	Yes	Yes	Yes	No	No	7.6	124.1	3.1	10.0	54.9
	P17	Yes	Yes	Yes	Yes	No	No	4.9	286.4	16.9	15.0	75.0
	P18	Yes	Yes	Yes	Yes	No	No	165.4	208.0	69.7	14.0	65.1
	P19	Yes	Yes	Yes	Yes	No	No	247.1	635.6	22.2	16.0	70.6
Median								**102.7**	**204.1**	**16.5**	**14.0**	**65.1**

(Abbreviations: NA-not applicable, VAS-visual analog scale, SCORAD-SCORing Atopic Dermatitis)

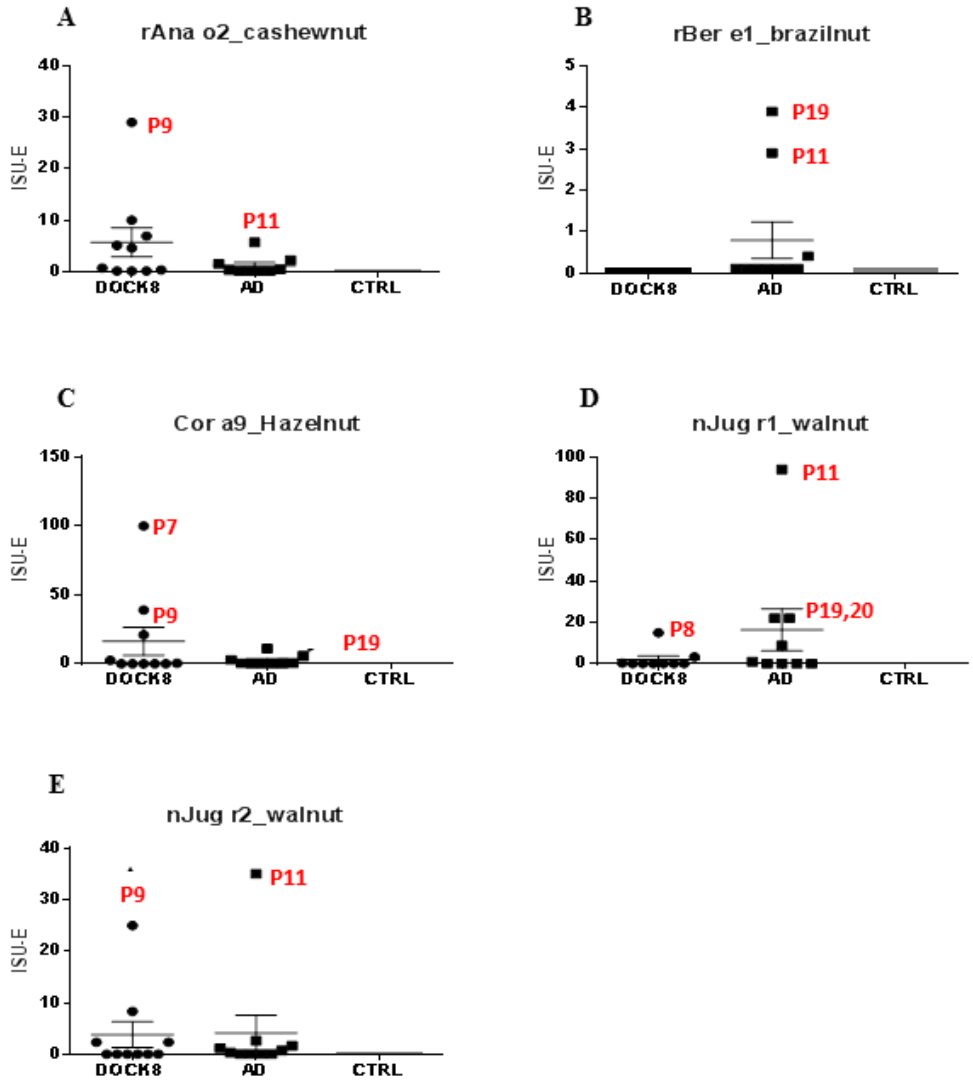

Figure 0.4: Treenut allergen sensitization pattern is not significantly different between DOCK8(n=10),atopic dermatitis(n=9)patients and ctrl(n=3).(A-E)
p-value< 0.05, P1- P10 are DOCK8-deficient, while P11-P19 are AD patients.
Abbreviations: AD-Atopic dermatitis, Ctrl-controls

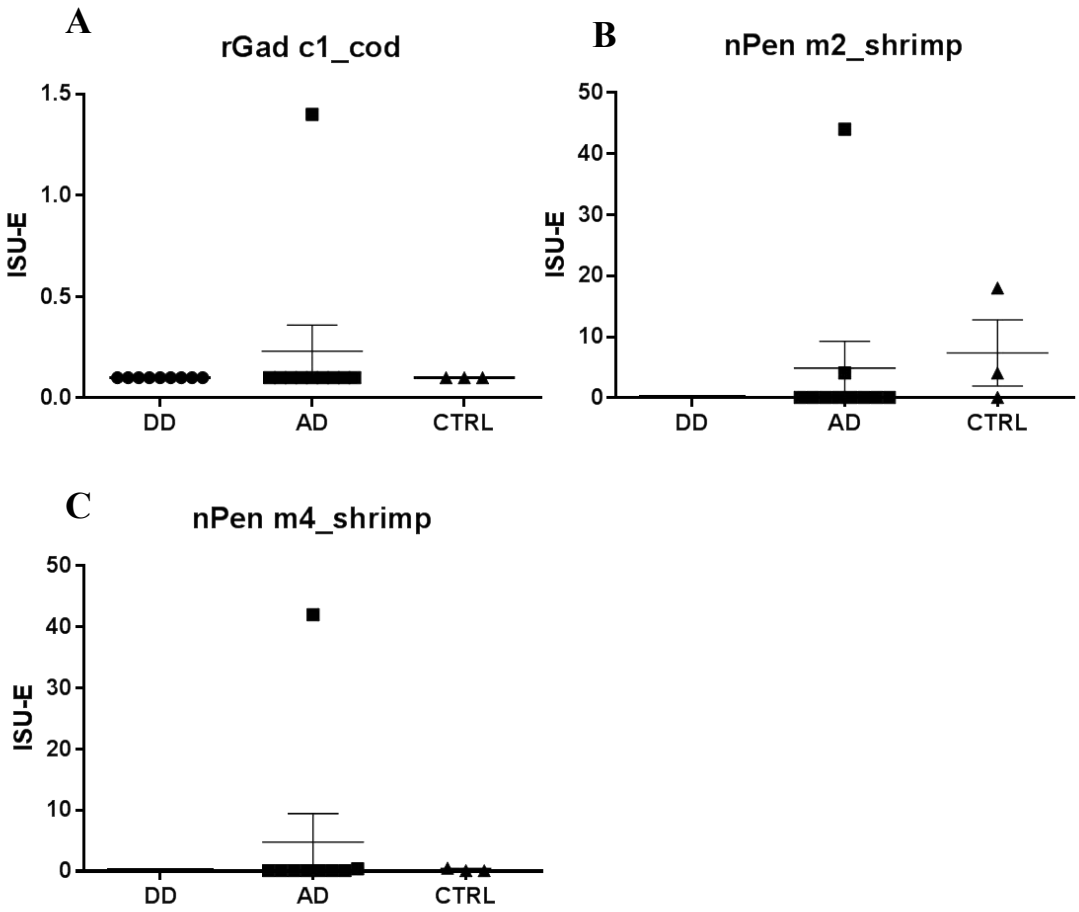

Figure 0.5: Sensitization profiles for Shellfish between DOCK8-deficient (n=10) ,atopic dermatitis(n=9)patients and ctrl(n=3) shows no significant difference.(A-C) p- value< 0.05, P1-P10 are DOCK8-deficient, while P11-P19 are AD patients, Abbreviations: AD-Atopic dermatitis, Ctrl-ctrls.

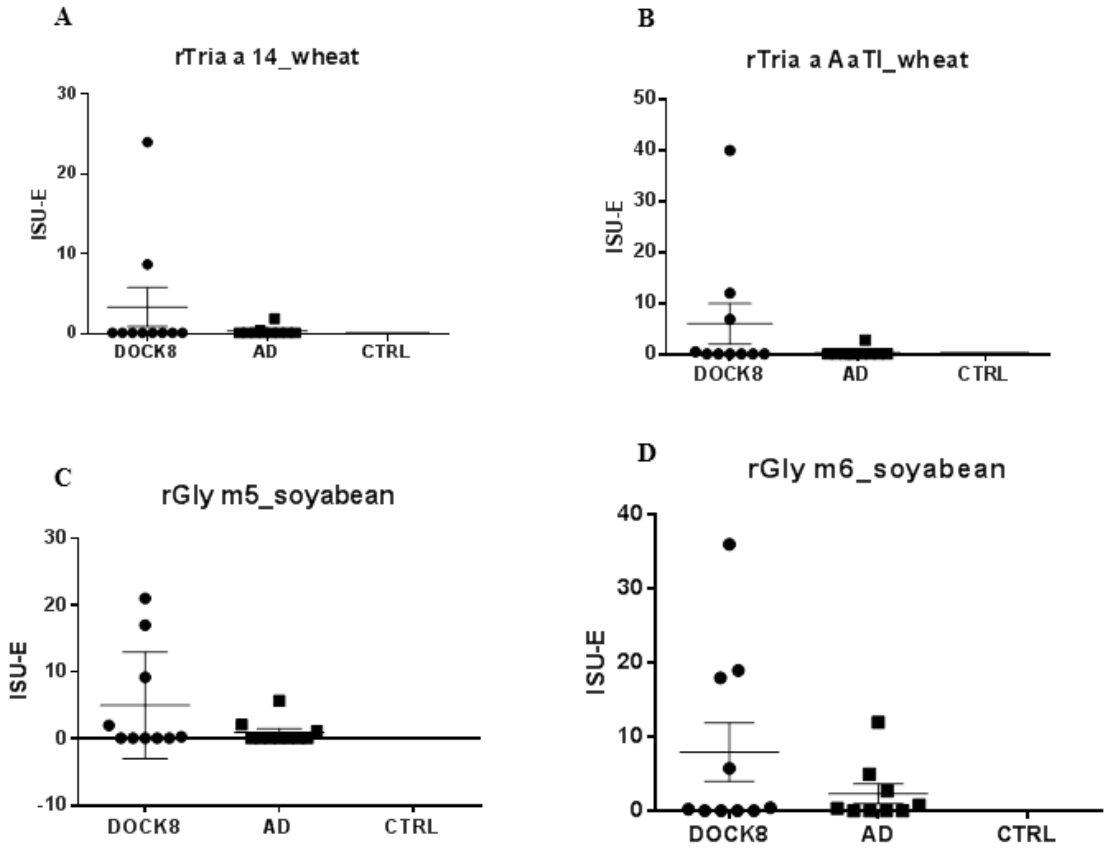

Figure 0.6: Wheat (A-B) and Soyabean (C-D) allergens sensitization pattern in DOCK8-deficient (n=10) and atopic dermatitis (n=9) patients using ISAC analysis. No stastical significance was observed in both the cohorts, p-value< 0.05, (Abbreviations: AD-Atopic dermatitis, ISAC-ImmunoCAP Solid-Phase Allergen Chip)

Noticeably among the aeroallergens, AD patients (78%) showed significantly higher sensitization to cat allergen rFeld1, compared to DOCK8-deficient patients (p-value<0.05). **(Figure 0.3H).** DOCK8-deficient patients showed moderate sensitization to latex (60%), plane tree (50%), and Timothy grass pollen (50%). AD patients also developed a higher sensitization to Bermuda grass (56%), Timothy grass (55%), Aspergillus (44%), Cypress (33%) and Cedar (33%) compared to DOCK8-deficient patients. Similarly, as seen among the food-allergens, the median and interquartile ranges (IQR) were comparable within the groups. (**Table A1.15)**. Three AD patients showed sensitization to cockroach and one AD patient (P15) showed sensitization towards dog, goosefoot and saltwort (P11), compared to no sensitization of these allergens in AD patients. Sensitization towards plane tree (50%) allergens was slighly higher in DOCK8-deficient (n=5) compared to AD (n= 4) patients.

5.4.4 Cytokine profiling

Cytokine/chemokine profiling for the same patients was performed for biomarker discovery as reported before.(6) Herein, we re-analyzed the same data based on the patients' food allergy profile. Multiple binary analyses were performed for IgE sensitization on the cytokines/chemokine data using the patient allergic vs. non-allergic status to a particular food or aero-allergen as a classifier. The significantly expressed cytokines/chemokines within each food group are summarized **Table 0.4.** In the DOCK8-deficient cohort, univariate analysis on cytokine profiling was performed between high and low sensitized allergen groups. For example, egg allergens (nGal d1 and nGal d3) profiling is represented in **Figure 0.7A-F**. IL-4 was up-regulated, in contrast to Scd40L (Soluble CD40-ligand) being down-regulated in high sensitized nGal d1 allergen group, whereas CCL11 and colony-stimulating factor3 (CSF3) were down-regulated in high sensitized nGal d3 allergen group. Among the milk allergens

(nBos d5 and Bos d8), CCL11and CSF3 were downregulated in high sensitized group. (**Figure 0.8: A- F) S**imilarly, in peanut allergens (rAra h1), transforming growth factor (TGF-A) and fibroblast growth factor 2 (FGF-2) were upregulated in high sensitized group were observed. (**Figure 0.9 : A-C**) and in soybean allergen (rGlym5) CCL11 and CSF3 (**Figure 0.9: D-F)** were differentially expressed in DOCK8-deficient cohort. No significant cytokines were detected in the sesame food group for patients. Among the AD patients, significant cytokines/chemokines expression were observed only for peanut and sesame allergen sensitisation. FGF-2(**Figure 0.10 : A, B)** was significantly upregulated in IgE sensitized patients to peanut allergen (rAra h3), and IL-3(**Figure 0.10: C, D**) was seen to be significant in sesame allergens.

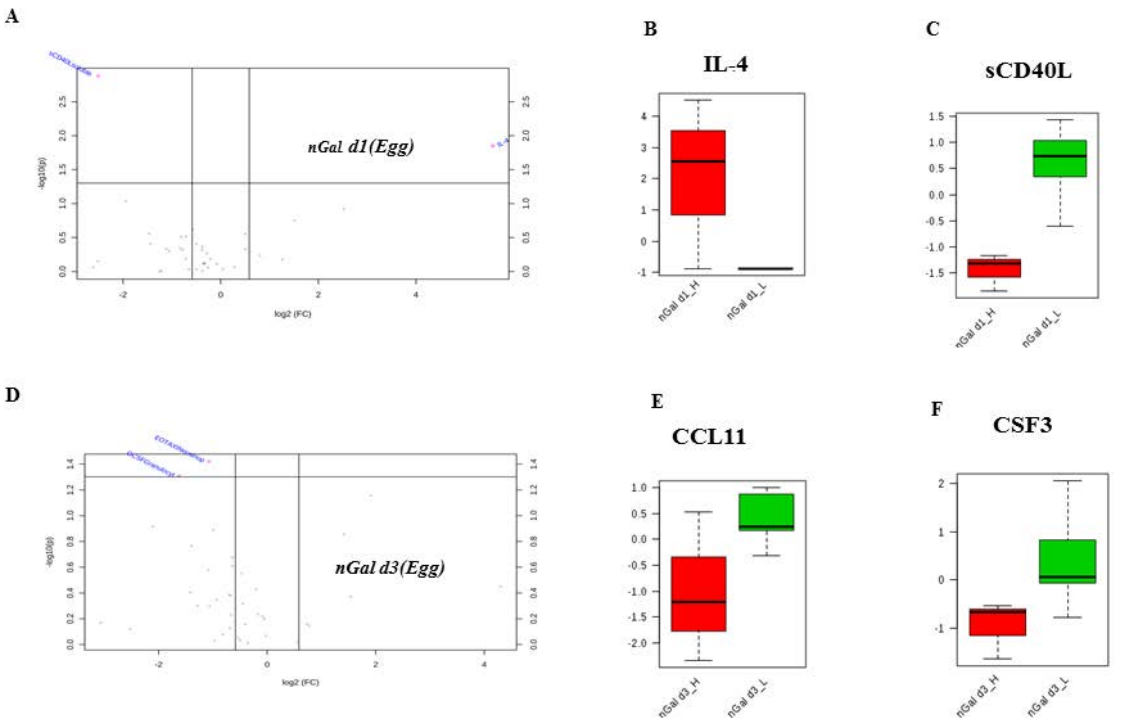

Figure 0.7: Sensitisation to specific egg allergens induces differential cytokine expression in DOCK8-deficient patients (n=10) (**A-C**) Volcano plots show IL-4 was up-regulated in association with nGal d1 sensitization(H) while Scd40l was down-regulated with nGal d1(L) (**D-F**)Volcano plots show CCL11 and CSF3 was down-regulated in association with nGal d3.Fold change=1.5, p-value<0.05

Table 0.4: Cytokine profiling summary of food-allergens in DOCK8-deficient and AD patients.

Allergen	Food Groups	DOCK8-deficient			AD		
		Patients	Significant cytokines	p-value	Patients	Significant cytokines	p-value
nGal d1	Egg	P2,P7,P8	IL-4 and Scd40l	0.03 and 0.003	P18,P19,P11	NS	NA
nGal d2		P2,P7,P8,P9	NS	NA	P18,P19,P11	NS	NA
nGal d3		P2,P7,P9	CCL11 and CSF3	0.03 and 0.04	P18,P11	NS	NA
nGal d5		P2	NA	NA	P11	NA	NA
nBos d4	Milk	P2,P8,P9	NS	NA	P18,P11	NS	NA
nBos d5		P2,P7,P9	CSF3 and CCL11	0.04 and 0.03	P18,P11	NS	NA
nBos d6			NA	NA		NA	NA
nBos d8		P2,P7,P9	CCL11 and CSF3	0.03 and 0.04		NS	NA
rAra h1	Peanut	P1,P7,P9	TGFA and FGF-2	0.02 and 0.04	P18,P19,P11	NS	NA
rAra h2		P8,P2,P9	NS	NA	P18,P19,P11	NS	NA
rAra h3		P1,P7,P2	NS	NA	P19,P11	FGF-2	0.04
rAra h6		P8,P2,P9	NS	NA	P18,P19,P11	NS	NA
rGly m5	Soybean	P2,P7,P9	CCL11 and CSF3	0.03 and 0.04	P18,P19,P11	NS	NA
rGly m6		P2,P5,P9	NS	NA	P18,P19,P11	NS	NA
nSes i1	Sesame	P2,P7,P8,P9	NS	NA	P18,P19,P11,P12	IL-3	0.04

(Abbreviation: CSF3-colony stimulating factor, FGF-2-fibroblast growth factor, TGFA-transforming growth factor,AD- Atopic dermatitis,NS-Not significant,NA-Not applicable)

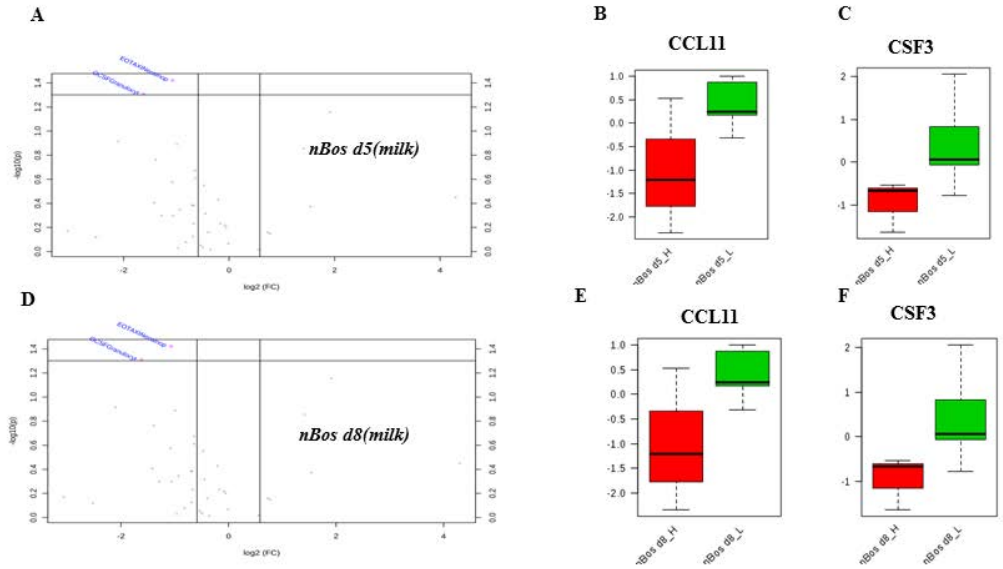

Figure 0.8: Milk allergy is associated with down-regulated expression of cytokines CCL11and CSF3 in DOCK8-deficient patients. (n=10) (A-F)

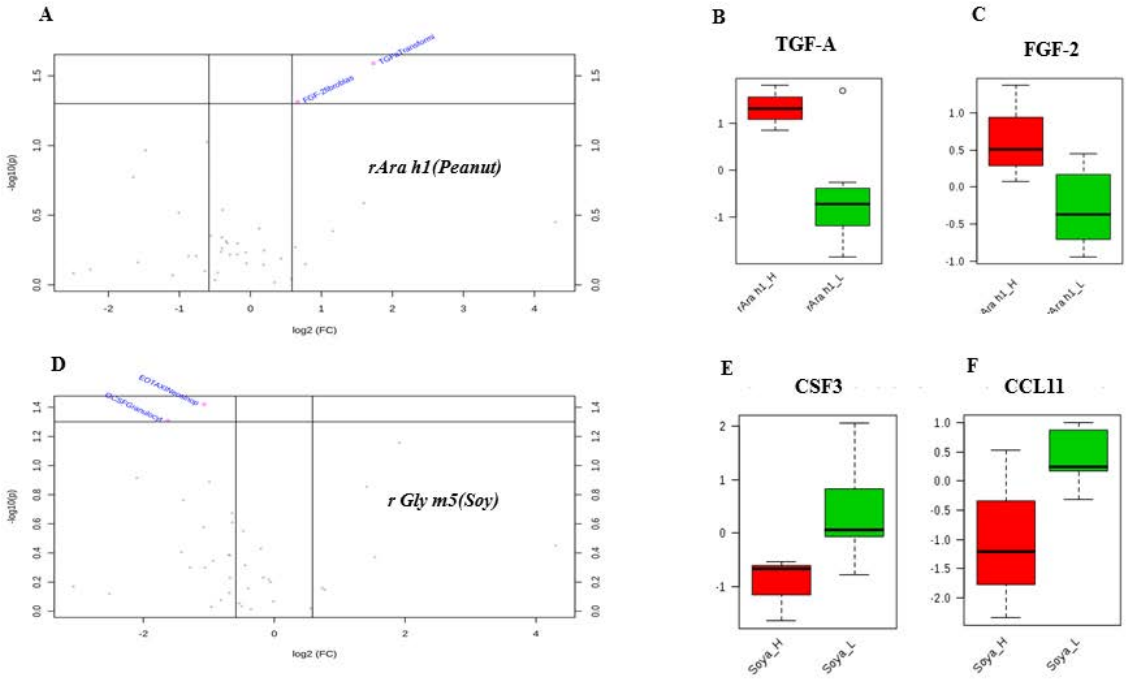

Figure 0.9 : Peanut and soy allergy in DOCK8-deficient patients(n=10) induces up regulation of cytokines TGF-A and FGF-2 and down regulation of CSF3 and CCL11

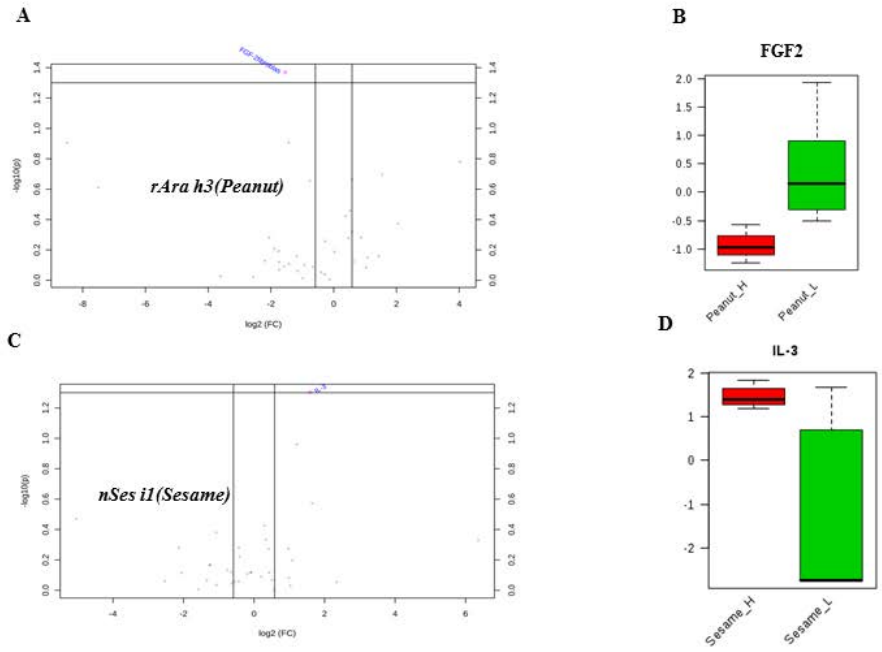

Figure 0.10: Peanut and Sesame allergens induce down regulation of cytokine FGF2 and up regulation of IL-3 expression in atopic dermatitis patients (n=9).

5.4.5 Metabolomics profiling:

Metabolomics profiling for our patients was also performed for biomarker discovery as discussed in chapter 4.(7) Multiple binary analyses were performed on each disease group, sub classified based on certain IgE binding to food-allergens (i.e. DOCK8-deficient allergic to egg vs DOCK8 deficient patients not allergic to egg). The metabolomics profile for the significant food-allergens, (egg, milk, peanut, sesame and soybean) were analyzed using MetaboAnalyst software version 3.0(http://www.metaboanalyst.ca) as described before in the method section.

In the DOCK8 cohort, the metabolomics binary comparison for egg allergen sesitisation showed cluster separation as demonstrated for sPLS-DA (**Figure 0.11A),** and the volcano plot showed 17 significantly expressed metabolites. (11downregulated and 6 upregulated) (**Figure 0.11B)** The same analysis revealed a distinctive metabolic profile for each food allergen such as milk (6 downregulated and 1 upregulated) (**Figure 0.12 : A-B**), peanuts (10 downregulated and 6 upregulated) (**Figure 0.13: A-B**), sesame (10 downregulated and 15 upregulated) (**Figure 0.14: A-B)** and soy (6 downregulated and 2 upregulated). (**Figure 0.15: A-B)** Significantly, expressed metabolites in the DOCK8-deficient cohort, for five food groups, egg, milk, peanut, sesame and soy, are represented in the loading plot. (**Figure 0.16 A-E)**

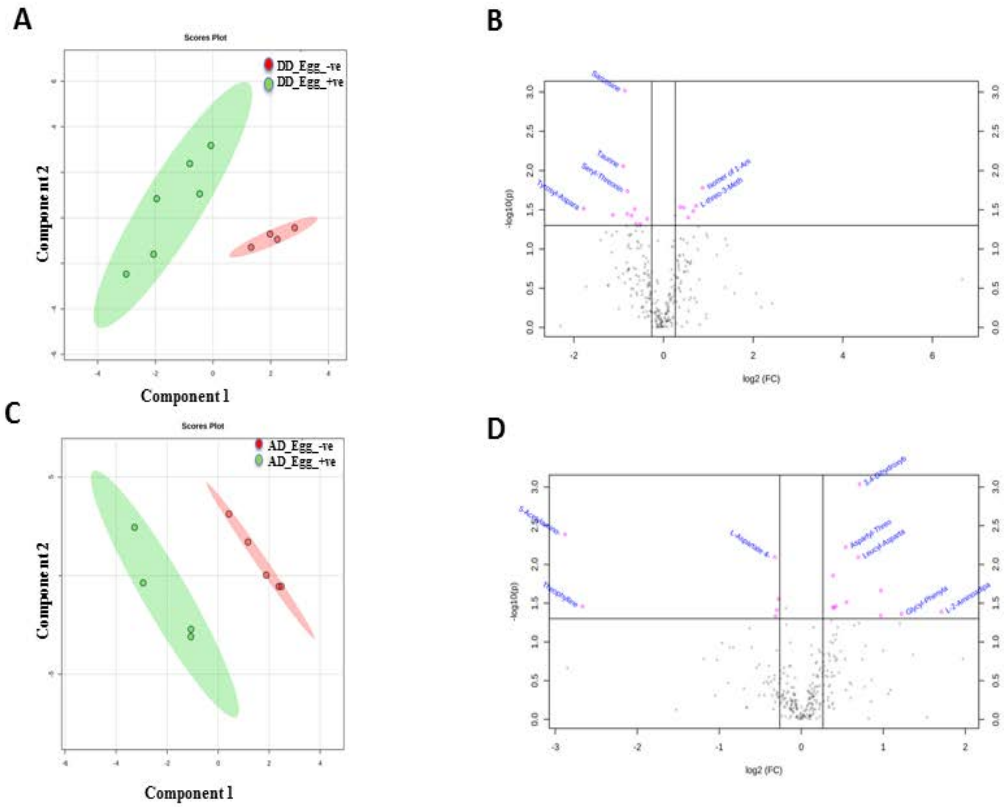

Figure 0.11:sPLS-DA score plot analysis for significant metabolites in DOCK8-deficient (n=10) and atopic dermatitis (n=9) patients with(+) and without(-) egg allergy (**A,C**) with the corresponding volcano plots respectively.(**B,D**) Fold change=1.2, p-value<0.05{Abbreviations: AD-Atopic dermatitis, Sparse Partial Least Square Discriminant Analysis (sPLS-DA)}

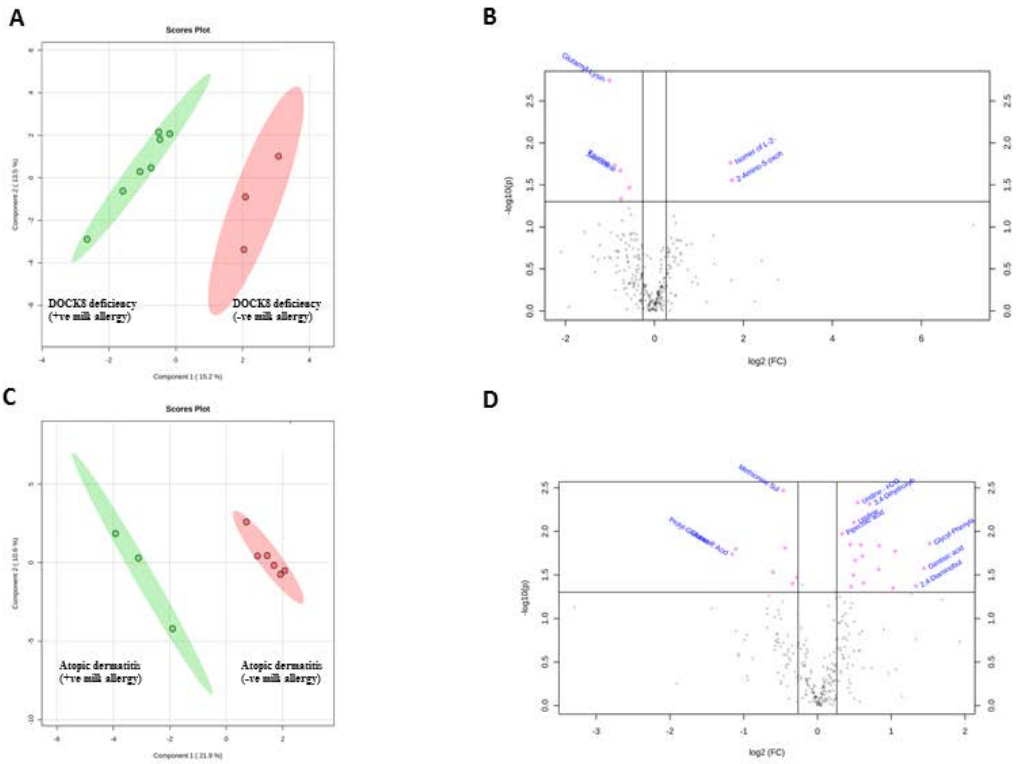

Figure 0.12: sPLS-DA score plot analysis for significant metabolites in DOCK8-deficient (n=10) and AD (n=9) patients with (+) and without (-) milk allergy (A,C) with their corresponding volcano plots respectively.(B,D) Fold change=1.2, p-value<0.05,{Abbreviations:AD-Atopic dermatitis, Sparse Partial Least Square Discriminant Analysis (sPLS-DA)}

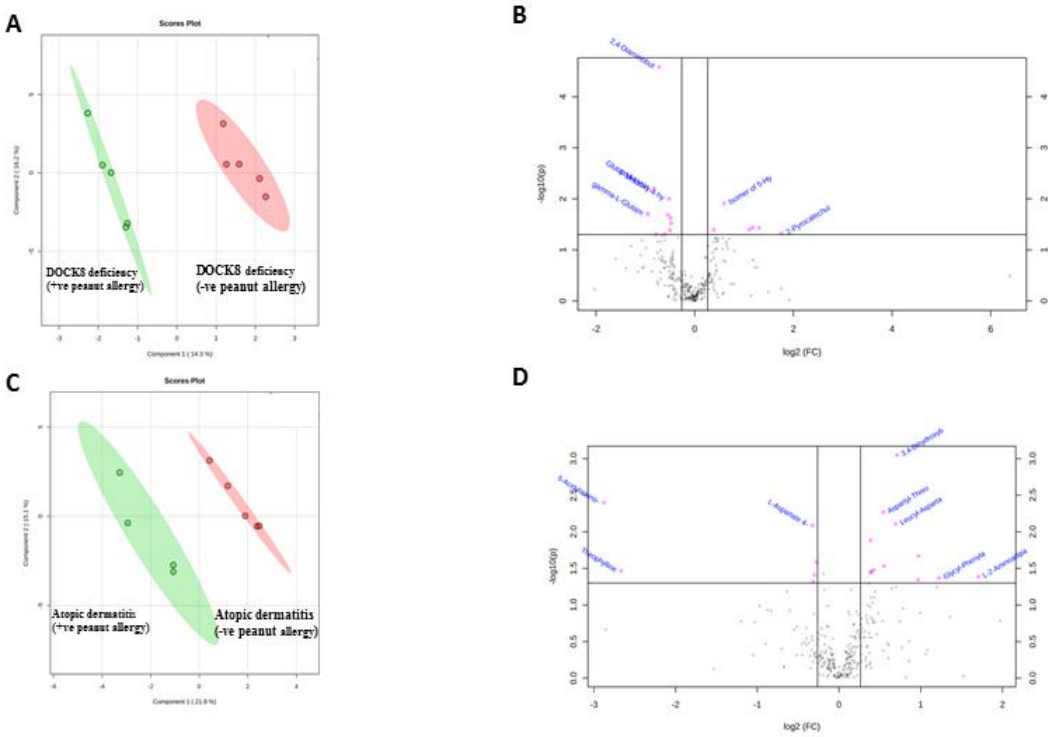

Figure 0.13: sPLS-DA score plot analysis for significant metabolites in DOCK8-deficient (n=10) and AD (n=9) patients with(+) and without(-) peanut allergy (A,C) with their corresponding volcano plots respectively.(B,D) Fold change=1.2, p-value<0.05,{Abbreviations:AD-Atopic dermatitis, Sparse Partial Least Square Discriminant Analysis (sPLS-DA)}

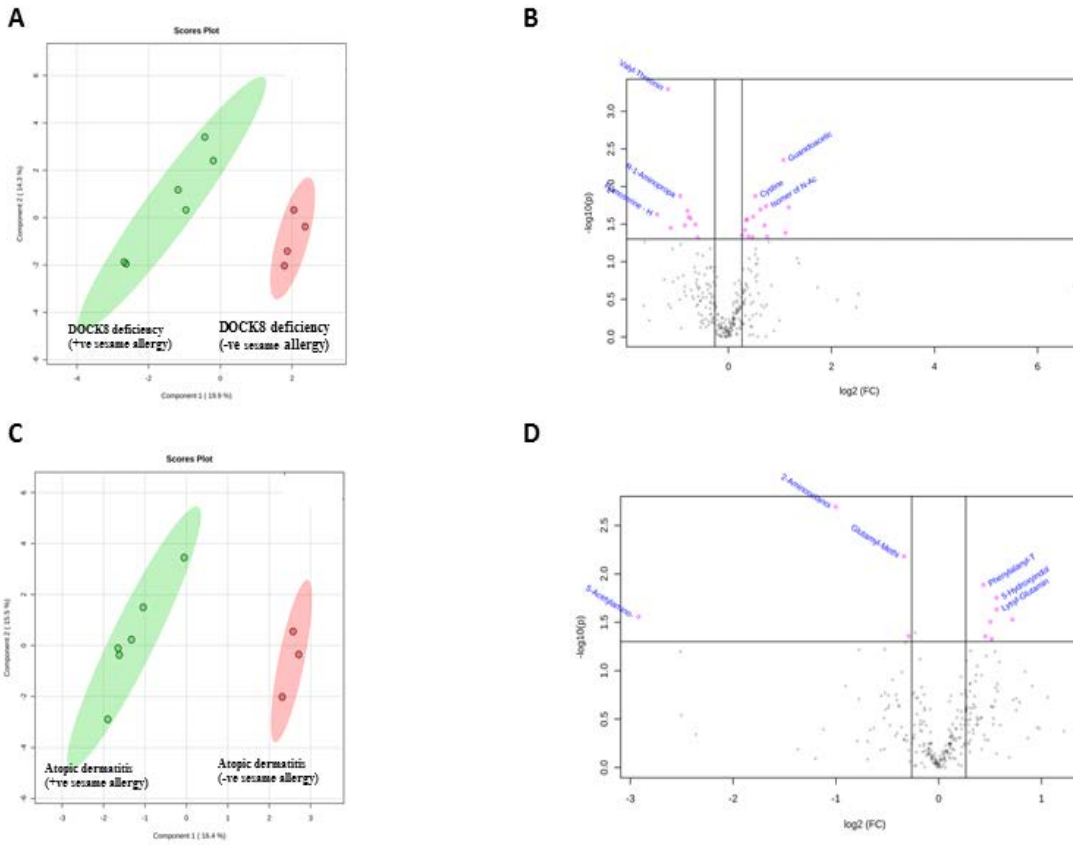

Figure 0.14: sPLS-DA score plot analysis for significant metabolites in DOCK8-deficient (n=10) and AD (n=9) patients with(+) and without(-) sesame allergy (A,C) with their corresponding volcano plots respectively.(B,D) Fold change=1.2, p-value<0.05,{Abbreviations: AD-Atopic dermatitis, Sparse Partial Least Square Discriminant Analysis (sPLS-DA)}

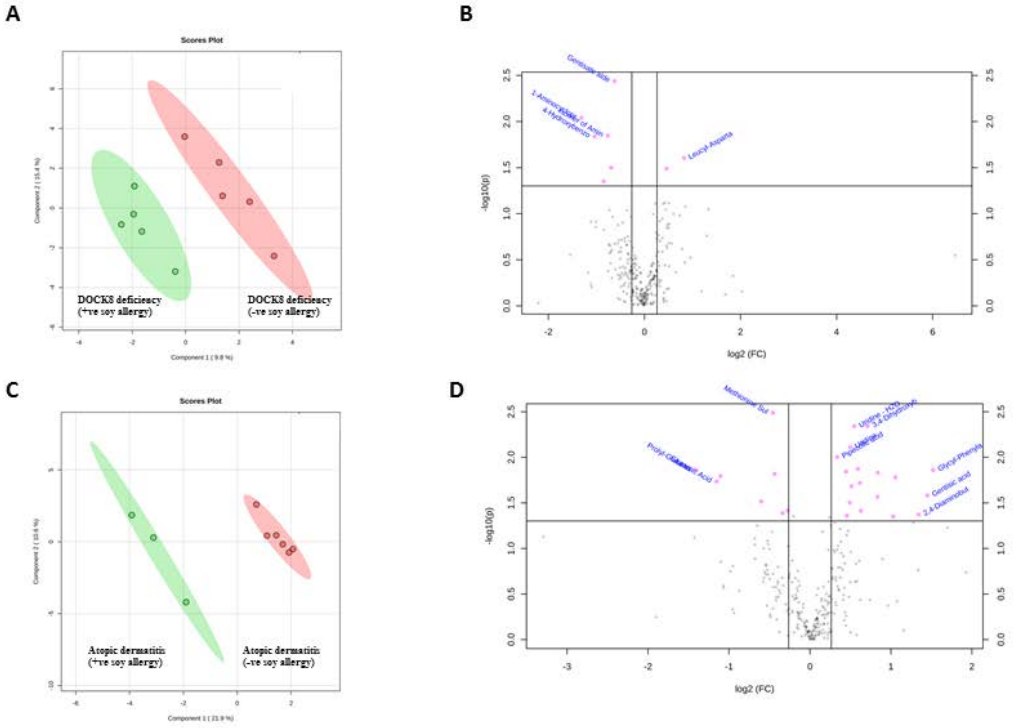

Figure 0.15 : sPLS-DA score plot analysis for significant metabolites in DOCK8-deficient (n=10) and AD (n=9) patients with(+) and without(-) soy allergy (A,C) with their corresponding volcano plots respectively.(B,D) Fold change=1.2, p-value<0.05,{Abbreviations: AD-Atopic dermatitis, Sparse Partial Least Square Discriminant Analysis (sPLS-DA)}

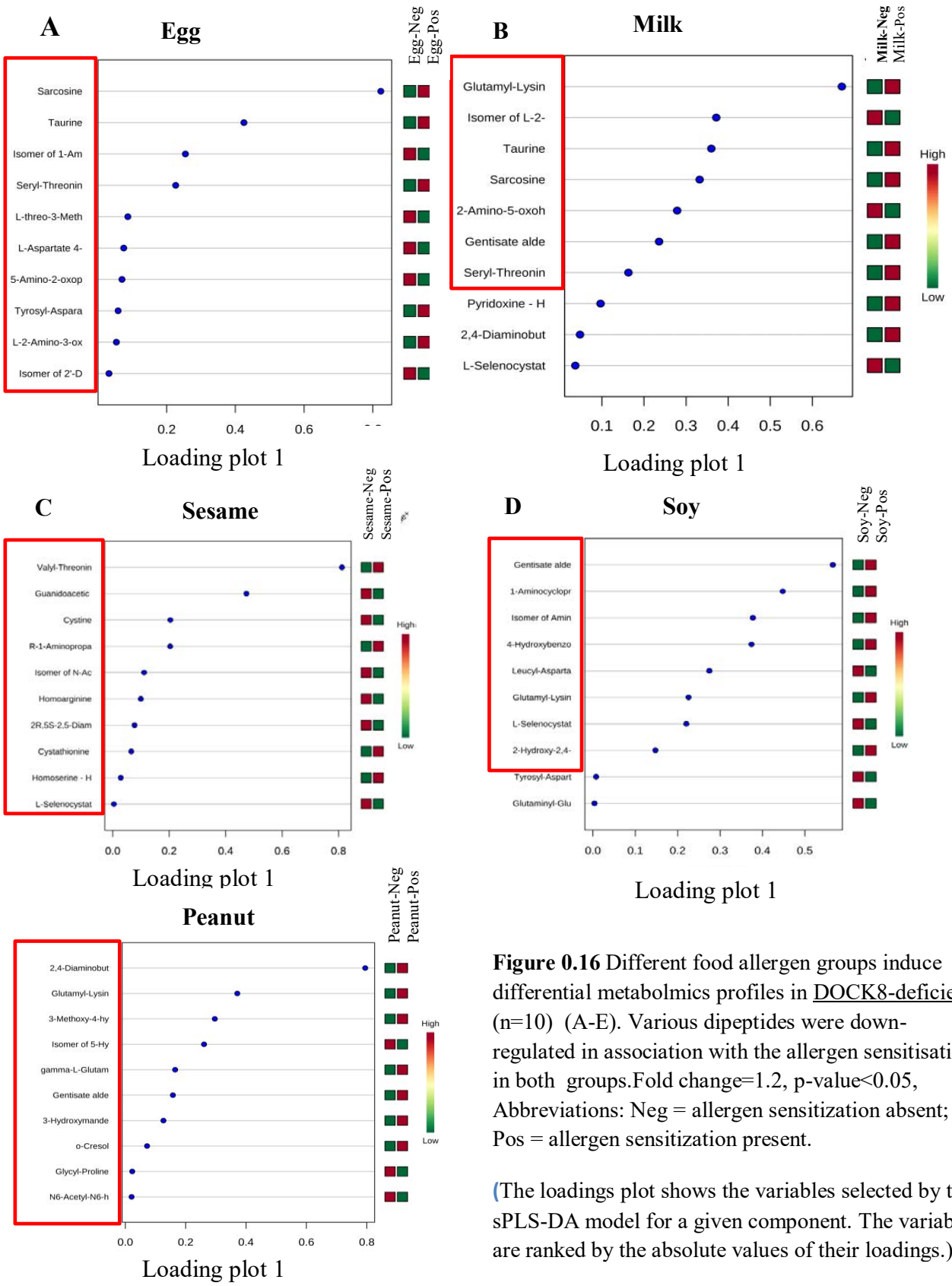

Figure 0.16 Different food allergen groups induce differential metabolmics profiles in DOCK8-deficient (n=10) (A-E). Various dipeptides were down-regulated in association with the allergen sensitisation in both groups.Fold change=1.2, p-value<0.05, Abbreviations: Neg = allergen sensitization absent; Pos = allergen sensitization present.

(The loadings plot shows the variables selected by the sPLS-DA model for a given component. The variables are ranked by the absolute values of their loadings.)

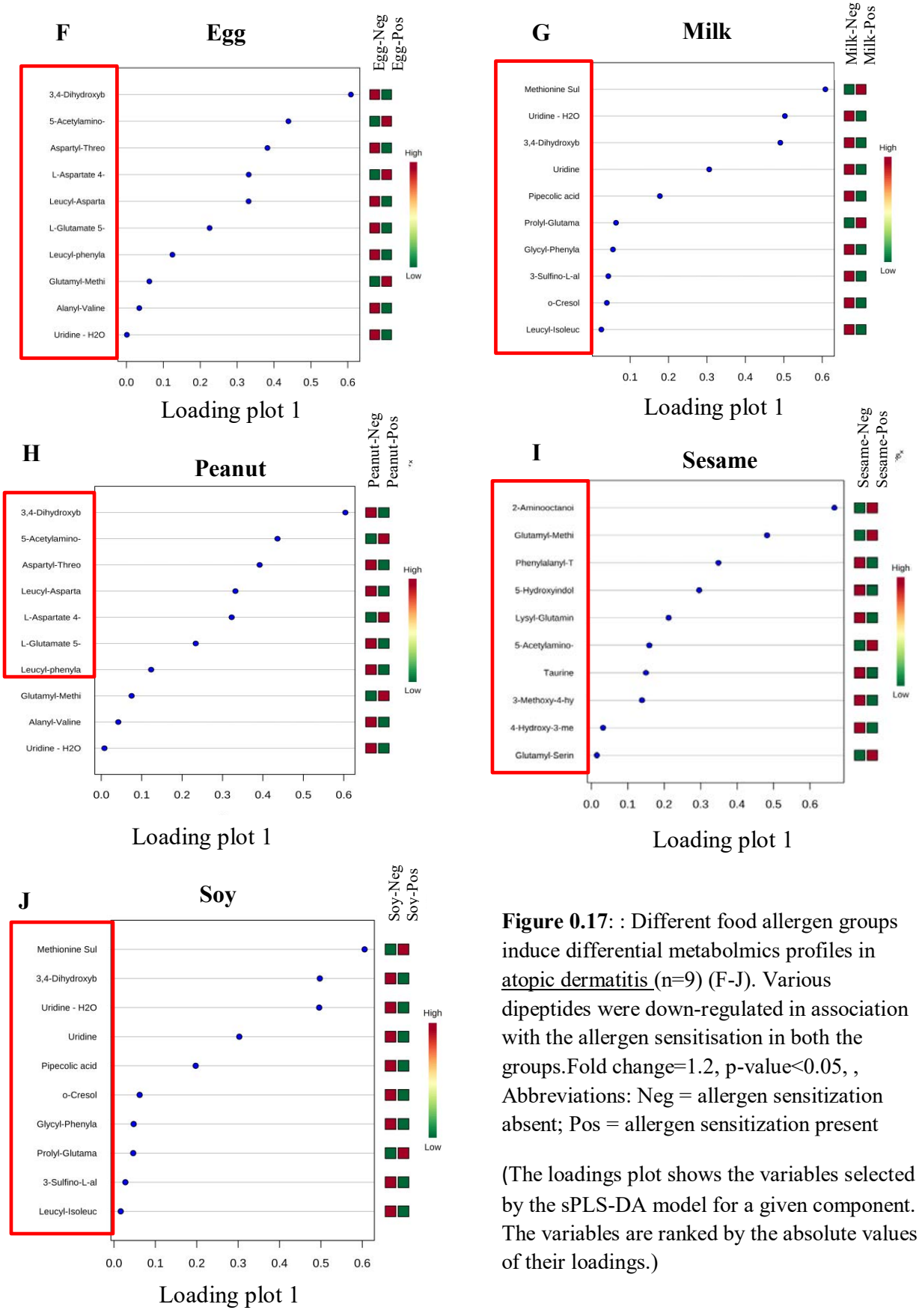

Figure 0.17: : Different food allergen groups induce differential metabolmics profiles in atopic dermatitis (n=9) (F-J). Various dipeptides were down-regulated in association with the allergen sensitisation in both the groups.Fold change=1.2, p-value<0.05, , Abbreviations: Neg = allergen sensitization absent; Pos = allergen sensitization present

(The loadings plot shows the variables selected by the sPLS-DA model for a given component. The variables are ranked by the absolute values of their loadings.)

Similarly, the AD cohort, showed cluster separation and is demonstrated in sPLS-DA**. (Figure 0.11C)** Volcano plot analysis showed 17 metabolites were significant, (7 down-regulated and 10 up-regulated). (**Figure 0.11D)** Similar analysis revealed a distinctive metabolic profile for each allergen group including milk (**Figure 0.12: C-D)** (9 down-regulated and 17 upregulated), peanut (**Figure 0.13: C-D)** (6 down-regulated and 12 up-regulated), sesame (4 down-regulated and 7 up-regulated) (**Figure 0.14 : C-D**) and soybean (9 down-regulated and 17 up-regulated). (**Figure 0.15: C-D)**. Significantly expressed metabolites, in the five food groups, egg, milk, peanut, sesame and soy are represented in the loading plot. (**Figure 0.17**: **F-I).**

For the five food groups, among the most significant differential expressed and disease-specific metabolites, 50 were identified in the DOCK8- deficient (**Table A1.16)** and 34 in the AD cohort (**Table A1.17)**. For example, in DOCK8- deficient cohort 2R, 5S-2, 5-diaminohexanoate is not disease-specific for any of the food groups whereas, in the AD cohort 3, 4-dihydroxybenzeneacetic acid and glutamyl-methionine are disease-specific in all the food groups.

Pathway analysis in DOCK8-deficient patients **(Figure A2 7**) showed dysregulation of several amino acid metabolism especially arginine, proline, glycine, serine and tryptophan, whereas in the AD cohort, the caffeine metabolism pathway was predominantly perturbed. Furthermore, pathway analyses stratified by the five food groups (egg, milk, peanut, sesame, and soy) were also performed in each cohort. (**Table A1.18**) As shown, different food-allergens induced different, non-overlapping- amino acid pathway dysregulation in both cohorts. However, the caffeine metabolism pathway induction correlated with egg and peanut allergy in patients with AD, taurine and hypotaurine metabolism pathway correlated with

milk allergy in DOCK8 deficiency and sesame allergy in atopic dermatitis. Tryptophan metabolism pathway induction was observed in association with egg and sesame allergy in the DOCK8 deficient patients only.

Discussion

Associations between food allergies, food sensitization and atopic eczema seem to be selective and population-based. The molecular mechanism behind the dramatic elevation in IgE levels in DOCK8-deficient patients is not clearly understood. Boos et al.(2014) examined the sensitization patterns in 6 DOCK8-deficient and 14 AD patients with 12 allergens (6 aero-allergens and 6 food-allergens) and demonstrated that the ratio of aero-allergen specific IgE to total IgE was the highest in AD, whereas DOCK8-deficient patients showed the highest specific serum IgE sensitization towards food-allergens.(12) Furthermore, among the food-allergens, higher sensitization was seen in DOCK8-deficient patients towards milk, whereas in AD patients towards cat allergen (12), which is in agreement with our findings. Moreover, in our study, we targeted 112 allergens compared to 12 allergens in theirs.

As shown in the study by Boos et.al (2014), our DOCK8-deficient patients also showed the highest sensitization towards cow milk allergens, particularly nBosd4, nBosd5, and nBos d8, as compared to AD patients. Studies have shown that casein (nBos d8) and beta-lactoglobulin (nBos d5) serum IgE antibodies are known markers of milk allergy.(13, 14) Patients with IgE antibodies against nBos d8 are reported to be less likely to outgrow this allergy, although most milk allergic children outgrow their allergy and develop tolerance towards milk, the mechanism of this tolerance is not well understood. The natural tolerance acquisition could be due to the decrease in the difference between IgE and IgG binding intensity to cow milk epitopes.(15) Furthermore, higher IgE levels to the egg allergene Gal d1 are known risk

factor for egg allergy, whereas undetectable levels may indicate that these individuals can tolerate cooked eggs.(16) nGal d 1 is the dominant egg allergen which we also demonstrated in our DOCK8-deficient and AD cohorts. IgE antibodies that react against sequential epitopes give rise to persistent allergies whereas IgE antibodies that react with conformational epitopes give rise to temporary allergies. (17) Gal d2 is the most abundant but heat-labile allergen in egg. Children with specific IgE to nGal d 2 can tolerate heat-denatured forms of the egg which is reported as the third most common food to cause anaphylaxis.(18) Interestingly in a recent clinical trial, The Learning Early About Peanut Allergy (LEAP) demonstrated that the early introduction of peanut decreases the occurrence of peanut allergies in children at high risk.(19) Our DOCK8-deficient and AD patients also exhibited sensitization towards soy and sesame allergens. Similarities between peanut and soy allergens, have been shown through IgE epitope mapping which may explain the IgE cross-reactivity between peanut and soybean.(20) Sensitization towards soybean allergens like Gly m5 and Gly m6 can also lead to allergic reaction. Severe allergic reactions in Japanese children have been reported towards soy allergens. (21) Allergic sensitisation towards sesame allergens are higher in the Middle East regions, and especially in Saudi Arabia sesame is reported as the third most common food to cause anaphylaxis.(22) Allergies towards peanuts, soybean, egg, and sesame, commonly seen in our cohort could be also due to the ethnic differences in food exposures and consumptions.

Among the aero-allergens, DOCK8-deficient patients showed high sensitization towards latex (Hev b1, 3) seems to be clinically irrelevant because no association between allergens and severity of the reactions have been identified so far.(23, 24) Studies from KFSH&RC in Saudi Arabia have also shown that latex allergy was observed in 3.4% of study subjects. (22) Grass pollen is the most common cause of seasonal respiratory allergy affecting the general

population worldwide.(25, 26) In a Spanish study, 99.4% patients allergic to grass pollen were polysensitized to unrelated pollens with rPhl p1, rPhl p5, rPhl p7 and rPhl p12. In our study, an average of 50% of sensitization was seen for rPhl p1, rPhl p4 and rPhl p5in both cohorts. Palao et.al (2016) showed in their study that there was no relationship between the molecular sensitization profile of these allergens and clinical features of respiratory allergy in their population. (27) DOCK8-deficient patients also showed higher sensitization towards olive allergen, nOle e9 compared to AD patients. Olive tree is a main source of allergy in the Mediterranean basin like Saudi Arabia and the prevalence of this allergen is dependent on the geographic area of sensitization. (28)Studies have shown that the in high in areas where the pollen counts reach up to 5000 per m^3 (certain regions of Andalucía and Italy) Ole e9 is highly prevalent allergen with sensitization rates of 50-60% among the allergenic population. (29) The above finding reflect that AD patients show more sensitization to inhaled allergens as compared to the DOCK patient

However, DOCK8-deficient patients showed higher sensitization towards olive, nOle e9, as compared to AD patients. Olive tree is a main source of allergy in the Mediterranean basin including Saudi Arabia and the prevalence of this allergen is dependent on the geographic area of sensitization. (28) Studies have shown that sensitization is high in areas where the pollen counts reach up to 5000 per m^3(certain regions of Andalucía and Italy) Ole e9 is highly prevalent allergen with sensitization rates of 50-60% among the allergic population.(29) In our study, 40% DOCK8-deficient patients were sensitized to Ole e9 as compared to only 20% in the AD cohort.

Most of our AD patients suffered from asthma and showed high sensitization towards cat allergen, Fel d1 particularly, compared to no sensitization among the DOCK8-deficient

patients. Gronlund et.al (2008) have shown that higher levels of IgE antibodies against Fel d1 were associated with asthma in cat allergic individuals. (30) which we observed in our AD cohort. IgE reactivity against aeroallergens was in general lower in our DOCK8-deficient patients, the difference in disease severity between both cohorts alone does not explain the different sensitization patterns,indicates towards a more specific role of food allergy in the pathogenesis of DOCK8 deficiency.

Furthermore, among the cytokines analysed in the DOCK8 cohort, dysregulated expression of CCL11 and CSF3 were commonly seen in egg, milk, and soybean sensitisation. CCL11 has been demonstrated to play an important role in inflammatory responses and the pathophysiology of food allergies in asthma and AD. (31) In the intestinal epithelial cells, CCL11 is also known to restrict the severity of allergic responses to food antigens.(32) The significance of this cytokine in the DOCK8 deficient cohort seems to be associated with the clinical profiles of these patients, inclusing asthma, infections and food allergies. While CSF3 is a glycoprotein linked to proliferation and maturation of neutrophils, the role in cancer development has been shown.(33) Scd40L and IL-4 were significant upregulated cytokines seen for egg sensitization in our study. Scd40L is known to act as an immunosuppressant in HIV infections, (34) whereas studies have shown that IL-4 favors food allergy by blocking the triggering of allergen specific Treg cells.(35) Significant expression of TGF-A and FGF2 was also observed in peanut sensitization. These growth factors are involved in many biological processes including, embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion.

Among the AD patients also FGF2 was also significantly expressed in peanut allergen, whereas IL3 was significant in sesame allergen, IL3 is known to play a critical role in

modulating innate immune effector functions in humans.(36) Taken together, significant expression of these cytokines and growth factors supports the role of pathogenic immune responses including food allergies, infections, favoring tumor growth and progression which is in agreement with disease phenotypes seen in the DOCK8 deficient and AD cohorts.

Metabolomics profiling in DOCK8-deficient patients revealed, aminoacid metabolism pathways to be commonly dsyregulated among the different food groups. Glycine and phenylalanine are known to play an essential role in cell signaling by impacting specific amino acid degradation pathways.(37).Hypotaurine behaves as an antioxidant and protective agent under physiological conditions, whereas under hypoxic signaling, hypotaurine behaves as an onco-metabolite promoting tumor progression.(38) Mammals are known to oxidizehypotaurine to taurine using trace amounts of hydrogen peroxide (H^2O^2) produced by cellular metabolism, which is likely to be more frequent in the brain than the liver. Alterations in tryptophan metabolism was seen among DOCK8 cohort, which confirms our previous study. (8) and it may be disease specific and not allergy specific. Hence the alterations in tryptophan metabolism seen in our cohorts reflect upon the pathophysiology of the disease, in relation to infections during the course of the disease.

In AD patients, the most significantly altered pathways among the five food groups was tyrosine metabolism, caffeine metabolism, and glutamine & glutamate metabolism. Tyrosine is a precursor to catecholamine compounds like dopamine, norepinephrine, and epinephrine. (39) released by the body in response to stress and was specific in AD patients only, hence it seems allergy specific and not disease specific. Glutamine plays an important role in the maintenance of gut mucosal integrity and function. Changes in intestinal permeability through cytokine release in response to food-allergens could manifest into food allergy.(40)

The Caffeine metabolism pathway was shown to be perturbed in egg and peanut food groups. Caffeine (1, 3, 7-trimethylxanthine) is a competitive antagonist of neurotransmitter adenosine or adenosine receptors, (42) that play an important role in many functions including regulation of neutrophils and their degranulation, ultimately having an effect on immunity. (43, 44) Merve et.al (2018) showed that caffeine inhibits STAT1 signaling and suppresses gene expression of pro inflammatory genes and cytokines that play an important role in autoimmune diseases.(45) Generally among both the cohorts perturbations in amino acid metabolism was observed and these changes have been reported in some cancers as well as neurodegenerative disorders such as Alzheimer disease and Parkinson diseases (46-48)

In summary, the differences in the comprehensive sensitization patterns in DOCK8-deficient and AD patients, were studied leveraging metabolomics and cytokine profiling, which revealed novel insights into the complexity of the immune and allergic responses that characterize DOCK8 deficiency (a rare inborn error of immunity) and atopic dermatitis (a more common human disease).Such studies will likely reveal additional molecular heterogeneity underpinning the varying disease severity and clinical responses to various therapeutic interventions as observed clinically.

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5.6 Chapter 5 summary

- Comprehensive ISAC based sensitization patterns were studied in DOCK8 deficient and atopic dermatitis patients
- The sensitization patterns were compared to metabolomics and cytokine profiling, revealing insights into the complexity of the immune and allergic responses that characterize DOCK8 deficiency and atopic dermatitis
- Such studies are likely to reveal additional molecular heterogeneity underpinning the varying dieases severity and clinical responses to various therapeutic inventions as observed clinically.

CHAPTER 6: GENERAL DISCUSSION AND FUTURE DIRECTION

Over the past decade, several studies have led to the hypothesis that DOCK8 deficiency might be a disease of impaired inflammatory response rather than a disease of pathologic IgE production. Elevated IgE levels, eczema, asthma, food, and environmental allergies are common or overlapping to both DOCK8 deficient and AD patients. If not treated with bone marrow transplantation, this condition may cause high morbidity and mortality. The DOCK8 protein deficient lymphocytes have difficulties migrating through the collagen gel matrices, which translates into high incidence of severe cutaneous viral infection commonly seen in these patients. Futhrermore, the molecular mechanism(s) underlying the significantly increased risk of cancer development in these patients is not clearly understood. So far, no definitive single or set of biomarkers have been identified, capable of distinguishing inherited DOCK8 deficient and AD patients.

The work outlined in this PhD thesis aimed at the development of a comprehensive metabolomics method involving a panel of 225 metabolites. Signature cytokine biomarkers were identified as outlined in Chapter 2 and metabolomics biomarkers are discussed in Chapter 4. Furthermore, in chapter 5 IgE sensitization pattersn to food and aero-allergens were studied and correlated with the cytokine and metabolomics profiles that were investigated in Chapters 2 and 4.

The panel of metabolites included in the metabolome represents a distinct metabolic fingerprint or signature, indicative of its current state. Finding metabolomic signature profiles can be useful in clinical care, biomarker discovery for several common and rare human diseases, and in understanding disease mechanisms and response to therapy. Metabolomics has influenced pharmacological research to a great extent and has made personalized health care increasingly possible. Metabolomics data are vast and complex; however, the use of multivariate analytical techniques for data processing and availability of high-quality analysis

software has made the data less complex. Thus, metabolomics is an essential component in biomarker discovery and its translation to clinical care strategies.

Comprehensive cytokine profiling revealed seven cytokines that were differentially expressed among the three comparative groups (DOCK8, AD and Ctrls). Interestingly, CXCL10 and TNF-A were the only signature cytokines, which were above the volcano plot's cutoff values and were significantly upregulated in DOCK8-deficient patients. CXCL10 is a chemokine, known to be highly expressed in various autoimmune diseases, pneumonia triggered by viral and bacterial infection as well as in asthma. Up regulation of this cytokine in the DOCK8-deficient cohort seems to be associated with the frequently occurring clinical phenotypes (asthma, infections, allergies, and pneumonia) seen in these patients. The higher circulating levels of CXCL10 in blood is due to either a higher competitive binding with a viral protein inflammation, (as it attracts the activated T lymphocytes, which are the only inflammatory cells expressing the chemokine receptor CXCR3), or DOCK8 inhibition of HS-GAG synthesis. In contrast, TNF-A is a multifunctional pro-inflammatory cytokine, known to mediate AD, psoriasis, tumor initiation and autoimmune diseases, and participates in cell survival/death and cancer. Up-regulation of TNF-A seems to contribute toward the pruritus and immune dysregulation observed in DOCK8 patients. In this study, EGF expression was significantly down-regulated in AD patients, but not in most DOCK8-deficient patients, when compared with controls, hence, low levels of EGF impair proliferation and anti-inflammatory immune response in the skin as has been demonstrated in several studies. IL-31 is a recently discovered cytokine produced by Th2 cells, and elevated levels of IL-31 are correlated with increased serum IgE levels and disease severity and are possibly responsible for the induction of pruritus in the AD patients.

An important approach for biomarker discovery is through understanding the metabolome, the ultimate functional product of the genome, which can be studied through identification and quantification of small molecules. The global metabolome influences the individual's phenotype in response to clinical and environmental interventions. The unique metabolic profiling can be useful in clinical care, biomarker discovery for several common and rare human diseases, as well as in understanding disease mechanisms and response to therapy. There is a strong clinical need to develop and validate quantitative methods capable of measuring the largest number of metabolites subject to wide and rapid dynamic changes present in a biological system. This is particularly challenging since several important factors such as metabolite dilution, stability, low abundance, sample matrix, concentration and availability of internal standards may significantly impact the ability to quantify these analytes. Development of an LC-MS/MS-based comprehensive targeted metabolomics panel, in Chapter 3, encompasses a broad range of clinical and biochemical genetic disorders and is applicable to different biological matrices such as whole blood, serum, dried blood spots (DBS) and tissues. The highlights of this methodology are the requirement of small sample volume, cost effectiveness and its utility in early and better management of these diseases. In this study, the effect of sample matrix on expression profiles was also investigated on a large set of clinically oriented metabolites, which may help to guide the choice of the optimal sample types and matrix in which it is collected.

A central objective of the experimental work conducted in this thesis, was identification of metabolomics biomarkers, using Chemical isotope labeling liquid chromatography-mass spectrometry (CIL LC-MS), capable of distinguishing DOCK8-deficient from AD patients. These biomarkers coupled with the identification of pathways potentially play an important role in the pathogenesis of DOCK8-deficient and AD patients and contribute towards improved disease monitoring and ultimately novel clinical interventions. It is critical to

recognize DOCK8 deficiency and differentiate its various clinical and molecular forms before severe life-threatening complications arise. Aydin, et.al (2015) in a study on 136 DOCK8-deficient patients, reported malignancies in 17%, life-threating infections in 58% and non-infections cerebral events in 10% of their patients. Krause et.al (2009) studied the neuroprotective activity of the tryptophan metabolite, 3-HAA, which may be helpful in future therapeutic approaches for neuroinflammatory disorders. Kyneurine pathway metabolomics profiling is dysregulated in the pathogenesis of many diseases characterized by inflammation and neurodegeneration, like Alzheimer's disease and Parkinson's disease. Metabolic finger printing studies by Rombust et.al (2017) has shown that 3-hydroxykynurenine and 3-hydroxyanthranilic acid can induce the formation of reactive oxygen species (ROS) under certain micro-environmental conditions, thereby playing a crucial role in the mechanisms underlying the relationship between consumption of red meat and associated diseases.

Aspartic acid is a major excitatory neurotransmitter and has been found to be increased in some epileptic and stroke patients. Since gut microbiota play important roles in host metabolism, especially in diabetes, Kai et.al (2019) found significantly altered metabolites, like aspartic acid, cholestan-3-ol (5β, 3α), and campesterol in association with lipogenesis and inflammation in pre diabetic mice. Furthermore, intraperitoneal injection of aspartic acid in mice has been found to have more beneficial effects on Experimental autoimmune encephalomyelitis, (EAE) widely used model for multiple sclerosis, reflecting upon the role of aspartate in therapy. In chapter 4, significant differential overexpression of 3-HAA and aspartic acid coupled with under expression of hypotaurine, guanosine, leucyl-phenylalanine, glycyl-phenylalanine, together seem to contribute to some of the immune and malignancy related phenotypes observed in this disease.

Furthermore, since both DOCK8 deficient and AD patients have food allergies in common, the cross reactivity sensitization pattern revealed difference in the sensitization patterns that

help understanding the allergic triggers, which may give some insight into disease manifestations and pathogenesis. Unique and shared cytokine and metabolomics alterations detected in this study reflect upon the distinct mechanisms functioning in these disorders. Although more and larger studies are needed, the existing study gives a broader picture of the sensitization patterns, providing information on specific and cross-reactive sensitizations that facilitate diagnosis, risk assessment and disease management.

Differentiating DOCK8-deficient patients from those with atopic diseases at an early stage is significant in a child's development, as treatment modalities differ considerably.

Naturally, arising somatic reversions of germ line mutations have been observed in several primary immunodeficiency disorders, including the Wiskott-Aldrich syndrome, severe combined immunodeficiencies, and X-linked lymphoproliferative diseases. Jing et.al (2014) showed that 50% of their DOCK8-deficient patients had somatic reversions and they were from a non-consanguineous background. This could be due to the DOCK8's location within a recombination hotspot that is characterized by many sub telomeric repetitive sequences. Such locations are known to contribute to large intragenic germ line deletions found in other human diseases, and contribute to the recombination-mediated somatic repair. In DOCK8 deficiency, only certain combinations of germline mutations support secondary somatic repair. Those patients had an ameliorated disease course with longer survival, but still had fatal complications or required hematopoietic cell transplantation.

In our cohort, none of DOCK8-deficient patients had somatic reversion and all came from a consanguineous background. Since all our patients had homozygous large deletions or compound heterozygous overlapping large deletion mutations, they were incapable of generating revertants, and are predicted to have a more severe form of the disease. In this

patient subgroup especially, HSCT is recommended to minimize the development of infection-related disease pathology.

Since DOCK8 deficient patient's shares clinical and laboratory features with AD patients, clinicians face a challenge of misdiagnosis in patients with DOCK8 deficiency. Elevated IgE levels, eczema, asthma, food, and environmental allergies are common to HIES and AD, although severe bacterial and viral infections, and pneumonia are exclusively seen in DOCK8-deficient patients. The cytokine and metabolomics profiling can add clinical value in distinguishing DOCK8-deficient patients from AD in timely fashion to start the treatment and management, while waiting for genetic confirmation. These biomarkers can be used in screening programs and follow up of post-transplanted patients. Pathway analysis insight can be useful to understand the pathophysiology of the disease and also used as a platform for future studies in the diagnosis of other types of HIES.

The outcome of this particular study reflects upon identification of differentiating biomarkers which may reveal insight, linking these disorders with their clinical phenotypes, thus facilitating improved understanding of disease pathogenesis, progression and better management.

Future direction:

The experimental data outlined in this thesis extensively characterizes major biomarkers differentiating DOCK8-deficent and AD patients, including the development of a comprehensive metabolomics method for biomarker discovery. The findings of this thesis provide a platform for the development of improved diagnostic approaches in biomarker discovery. Future advances in this field of research may be pursued in various aspects as follows:

a) Evaluation of the method using a larger patient cohort - The developed metabolomics panel requires small sample volume and is cost effective. However, an extensive panel with more metabolites needs to be investigated and validated in a larger patient cohort, so as to be used in clinical laboratories for the early detection and distinction of DOCK8-deficent and AD patients.

b) Evaluate additional biomarkers using other OMICs - Combining high throughput experimental "OMIC based" techniques such as genomics, proteomics, and metabolomics with computational techniques such as bioinformatics and computer simulations enable a better understanding of biomarker discovery. Differences within the proteome and dysregulation of protein expression aid in the identification of the perturbed pathways that might play a role in the immunological and pathological development of the disease thereby enabling therapeutic intervention. Future OMICs studies on these patient samples may help in the development of quantitative diagnostic markers to monitor disease progression or responses to therapy using proteomic and transciptomic approaches.

c) Biomarker evaluation in a cohort before and after stem cell transplantation - Apart from severe viral infection in DOCK8 deficient patients, high risk of malignancy is also reported in these patients and the only definitive and curative treatment option is hematopoietic stem cell transplantation. Current studies show that clinical findings, including eczema and food allergies, are resolved in majority of these patients after transplantation, although in many cases food allergies seem to persist, post transplantation (Aydin et.al, 2019).The patients recruited in this study were not transplanted. Future studies in investigating the metabolome and proteome, before and after transplantation are essential, and subsequent analysis of the changes will provide a detailed insight into the role of these immune related proteins and provide a deeper insight into the pathogenesis of the HEIS.

d) Evaluate specific food and aero-allergens from Saudi Arabia - The ISAC ImmunoCAP panel used in our Allergy Microarray has 112 antigenic epitopes and quantifies the IgE antibody concentrations in patient serum. However, this panel lacks common allergens seen in the Middle Eastern region including Saudi Arabia, including the edible fruit from dates (palm family) and other pollen allergens. There is a need to study the IgE sensitization pattern in the local population.

The experimental work and data analysis presented in this thesis generated substantial knowledge for the development of OMICs diagnostic platforms and is the foundation for the future development of key biomarkers in autoimmune diseases.

APPENDIX A: SUPPLEMENTARY TABLES AND FIGURES

Tables

Table A1.1: List of 39 Cytokine/Chemokines/Growth factors

Cytokines (n=22)
FLT3LG (FLT3L), IFN-A2, IFN-G, IL-10, IL12p70 (IL-12B), IL-13, IL-15, IL-17A, IL-1A, IL-1B, IL-1R1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, TNF(TNF-A) , LTA , IL-21
Chemokines (n=9)
CCL-2 , CCL-3 , CCL-4 , CCL-7, CCL-11 (Eotaxin),CCL22 (MDC), CX3CL1 (Fractalkine), CXCL1 (GRO), CXCL10 (IP-10)
Growth factor/ others (n=7)
EGF, FGF2, TGFA, CSF3(GCSF), CSF2 (GMCSF), sCD40L, VEGFA (VEGF)
IL31(n=1)
Human IL31 DUOSet Elisa kit

Table A1.2: List of metabolites and their optimized LC/MS parameters

Name of the metabolite	RT (min)	Internal standard	Pola-rity	Precuusor ion (m/z)	Product ion (m/z)	Cone voltage (V)	Collision ene-gy (CE)	HMDB	Pub-Chem	KEGG
17 α-Hydroxy progesterone	8.73	Ornithine-d6	Pos	331	97	65	20	00374	6238	C01176
2,3-Pyridine dicarboxylic (Quinolinic) acid	4.09	D-Fructose (2-13C)	Neg	166	122	20	11	0000232	16219881	C03722
21-Deoxycortisol	5.8	Alanine-d4	Neg	345	87	25	18	04030	222803	C05497
2-hydroxyglutaric acid	0.75	Alanine-d4	Pos	193	175	30	13	HMDB06213	4394846	C02917
2-Isopropyl malic acid	4.13	Citric acid-d4	Neg	175	115	32	16	HMDB00402	5280523	C02504
2-Ketobutyric acid	4.28	D-Fructose (2-13C)	Neg	101	57	52	8	HMDB00005	58	C00109
2-Phosphoglyceric acid	5.2	Valine-d8	Neg	187	131	25	18	HMDB00362	59	C00631
3,4-Dihydroxyphenyl acetic acid	3.57	D-Fructose (2-13C)	Pos	169	123	25	13	HMDB0001336	547	C01161
3-Hydroxyanthranilic acid	3.51	Alanine-d4	Neg	152	91	32	22	HMDB01476	86	C00632
3-Methylglutaric acid	4.16	D-Fructose (2-13C)	Neg	145	83	28	14	HMDB00752	12284	NA
3-Methylhistidine	0.86	D-Fructose (2-13C)	Pos	170	109	38	20	HMDB00479	64969	C01152
Methylmalonic acid	4.29	Methyl malonate-d3	Neg	117	73	30	12	HMDB00202	487	C02170
3-Phosphoglyceric acid	4.4	Valine-d8	Neg	185	79			HMDB00807	724	C00197
4,6-Dioxoheptanoic acid (succinylacetone)	3.49	D-Fructose(2-13C)	Neg	157	99	24	8	HMDB0000635	5312	NA
4-Hydroxy-L-glutamic acid	2.72	D-Fructose(2-13C)	Pos	163	100	20	15	HMDB02273	5460078	C03079
4-Hydroxyphenyl pyruvic acid	3.89	D-Fructose(2-13C)	Pos	179	107	20	10	HMDB00707	979	C01179
4-Hydroxyproline	2.01	Ornithine-d6	Pos	132	69	2	16	HMDB00725	5810	C01157
5,6-Dihydro-5-methyl uracil	1.62	Adenosine C13	Neg	127	42	17	15	HMDB00079	93556	C00906
5,6-Dihydrouridine	1.57	Adenosine C13	Neg	277	194	24	10	HMDB00497	94312	C00429
5-Aminolevulinic acid	1.1	Glucose-d7	Neg	130	112	30	12	HMDB01149	137	C00430
5-Hydroxyindoleaceti c acid	3.92	D-Fructose(2-13C)	Pos	192	146	25	15	HMDB00763	1826	C05635
5-Hydroxylysine	0.9	Alanine-d4	Pos	163	128	24	10	HMDB00450	3032849	C16741
6-Phosphogluconic acid	4.23	D-Fructose(2-13C)	Neg	275	97	68	19	HMDB01316	91493	C00345

Acetyl-CoA	5.61	D-Fructose(2-13C)	Neg	808	408	20	40	HMDB01206	444493	C00024
Adenine	1.47	Adenosine C13	Neg	134	107	24	24	HMDB00034	190	C00147
Adenosine	2.34	Adenosine C13	Neg	266	133.9	32	22	HMDB00050	60961	C00212
Adenosine diphosphate	4.6	Adenosine C13	Neg	426	133.88	48	24	HMDB01341	6022	C00008
Adenosine monophosphate	3.57	Adenosine C13	Neg	346	96.89	42	24	HMDB00045	6083	C00020
Adenosine triphosphate	5.41	Adenosine C13	Neg	506	158.83	48	46	HMDB00538	5957	C00002
Adenylsuccinic acid	4.96	Adenosine C13	Neg	462	134	62	46	HMDB00536	440122	C03794
Adonitol	1.1	D-Fructose(2-13C)	Neg	151	89	24	10	HMDB00508	6912	C00474
Alanine	0.94	Alanine-d4	Neg	90	72	20	6	HMDB0000161	5950	C01401
Aldosterone	4.86	Adenosine C13	Neg	359	199	15	17	HMDB00037	24758425	C01780
Aminoadipic acid	2.19	Leucine-d3	Neg	160	116	28	12	HMDB00510	469	C00956
Arabinose	1.03	D-Fructose(2-13C)	Neg	149	89	25	7	HMDB29942	66308	C00259
Argininosuccinic acid	2.27	D-Fructose(2-13C)	Neg	289	131	25	27	HMDB00052	16950	C03406
Betaine	1.28	Citrulline-d2	Pos	118	59	36	16	HMDB00043	247	C00719
Biopterin	1.38	L-Citrulline-d7	Neg	236	192	30	15	HMDB00468	445040	C06313
Biotin	4.46	Glucose-d7	Pos	245	227	50	25	HMDB00030	171548	C00120
Cholic acid	6.71	Citric acid-d4	Neg	407	343	78	42	HMDB00619	221493	C00695
Citrulline	1.02	Citrulline-d2	Pos	176	70	30	26	HMDB00904	9750	C00327
Coenzyme A	5.5	Alanine-d4	Neg	766	79	160	65	HMDB01423	6816	C00010
Corticosterone	7.69	Glucose-d7	Pos	347	121	48	24	HMDB01547	5753	C02140
Cortisone	6.82	D-Fructose(2-13C)	Pos	361	163	55	25	HMDB02802	222786	C00762
Creatinine	1	Ornithine-d6	Pos	114	44	20	13	HMDB00562	588	C00791
Cyclic AMP	3.54	Adenosine C13	Neg	327.8	133.9	46	22	HMDB00058	6076	C00575
Cyclic GMP	3.32	Adenosine C13	Neg	361.9	78.9	28	26	HMDB01314	24316	C00942
Cysteine-sulfate	2.72	D-Fructose(2-13C)	Neg	200	81	20	16	HMDB00731	115015	C05824
Cytidine	1.1	Adenosine C13	Neg	242	108.9	32	10	HMDB00089	6175	C00475
Cytidine mono phosphate-N-acetyl neuraminic acid (Sialic acid)	2.36	C2-Carnitine-d3	Pos	637	346	30	25	HMDB01176	448209	C00128
Cytidine monophosphate	3.1	Adenosine C13	Neg	323	96.9	38	22	HMDB00095	6131	C00055
Cytidine triphosphate	5.28	Adenosine C13	Neg	482	158.83	46	30	HMDB00082	6176	C00063
Cytosine	1.73	C5-Carnitine-d9	Pos	112	85	13	12	HMDB00630	597	C00380
Cytosine diphosphate	4.17	C12-Carnitine-d3	Neg	402	158.84	44	26	HMDB01546	6132	C00112
D-Arabitol	1.02	D-Fructose(2-13C)	Neg	151	89	30	10	HMDB00568	827	C01904
D-Aspartic acid	2.34	Ornithine-d6	Neg	132	88	27	13	HMDB06483	83887	C00402
Deoxyadenosine	4.8	Adenosine C13	Neg	250	133.9	30	16	HMDB00101	13730	C00559
Deoxyadenosine monophosphate	3.42	Adenosine C13	Neg	330	194.9	100	16	HMDB00905	12599	C00360
Deoxycytidine	1.7	Adenosine C13	Pos	228	112	25	15	HMDB00014	13711	C00881
Deoxycytosine monophosphate	2.79	Adenosine C13	Neg	306	78.8	36	24	HMDB01202	13945	C00239

Deoxyguanosine	1.54	Adenosine C13	Neg	266	149.9	40	16	HMDB00085	187790	C00330
Deoxyinosine	1.5	Adenosine C13	Neg	251	134.87	42	20	HMDB00071	65058	C05512
Deoxyribose-5'-phosphate	2.83	Adenosine C13	Neg	213	96.83	26	16	HMDB01031	45934311	C00673
Deoxyuridine	1.33	Adenosine C13	Neg	227	94	24	10	HMDB00012	13712	C00526
D-Erythrose-4-phosphate	3.07	C6-Carnitine-d3	Neg	199	97	21	8	HMDB01321	122357	C00279
Dexamethasone	5.76	D-Fructose(2-13C)	Neg	391	361	20	18	HMDB15364	5743	C15643
Dextrose	1.12	D-Fructose(2-13C)	Neg	179	89	25	10	HMDB00122	5793	C00031
D-Fructose	0.93	D-Fructose(2-13C)	Neg	179	89	16	8	HMDB00660	439709	C02336
D-Galactono-1,4-lactone	1.07	Glucose-d7		177	129	26	15	HMDB0002541	6857365	C01115
D-Glucose	0.95	Glucose-d7	Neg	179	89	25	10	HMDB00122	5793	C00031
D-Glucuronic acid	2.45	Glucose-d7	Neg	193	113	20	17	HMDB00127	444791	C00191
D-Glutamic acid	0.9	Glutamate-d5	Pos	148	84	24	12	HMDB03339	23327	C00217
Dihydrouracil	1.01	Alanine-d1	Neg	113	69	26	58	HMDB00076	649	C00429
Dihydroxyacetone phosphate	4.5	C16-Carnitine-d3	Neg	169	79	25	17	HMDB01473	668	C0011
Dimethylallylpyrophosphate (DMAPP)	4.96	C2-Carnitine-d3	Neg	245	74	34	16	HMDB01120	647	C00235
DL-Homocystine	1.14	Inosine-15N4	Pos	269	136	30	10	HMDB00575	10010	C01817
D-Maltose	1.02	Glucose-d7	Neg	341	101	30	20	HMDB00163	10991489	C00208
D-Mannose	0.95	Glucose-d7	Neg	179	59	25	17	HMDB00169	18950	C00159
D-Mannose-1-phosphate	2.73	Glucose-d7	Neg	259	79	25	20	HMDB06330	644175	C00636
Dopamine	1.06	C14-Carnitine-d3	Pos	154	91	26	19	HMDB00073	681	C03758
D-Ribose-5-phosphate	2.72	D-Fructose(2-13C)	Neg	229	97	25	15	HMDB01548	439167	C00117
D-Threitol	2.51	D-Fructose(2-13C)	Pos	123	105	30	8	HMDB04136	169019	C16884
D-Tryptophan	3.97	Ornithine-d6	Pos	205	146	30	18	HMDB13609	9060	C00525
Dulcitol	1.15	Inosine-15N4	Neg	181	101	50	15	HMDB00107	11850	C01697
D-Xylitol	1.04	Alanine-d4	Pos	153	117	22	8	HMDB02917	6912	C00379
D-Xylose	0.99	D-Fructose(2-13C)	Neg	149	89	30	15	HMDB00098	135191	C00181
Estradiol	7.98	C8-Carnitine-d3	Pos	273	186	25	24	HMDB00151	5757	C00951
Estriol	4.64	Alanine-d4	Neg	287	145	25	40	HMDB00153	5756	C05141
Estrone	8.36	Alanine-d4	Pos	271	133	40	20	HMDB00145	5870	C00468
Ethanolamine	0.84	Ornithine-d6	Pos	62	44	10	12	HMDB00149	700	C00189
Fructose-1,6-bis phosphate	4.56	D-Fructose(2-13C)	Neg	339	97	34	19	HMDB01058	172313	C00354
Fructose-6-phosphate	2.69	D-Fructose(2-13C)	Neg	259	197	30	15	HMDB00124	69507	C00085
Fumaric acid	6.1	C4-Carnitine-d3	Neg	115	71	30	6	HMDB00134	444972	C00122
Galactose-1-phosphate	2.81	D-Fructose(2-13C)	Neg	259	79	25	20	HMDB00645	123912	C00446
Gamma-Amino Butyric acid (GABA)	0.98	Alanine-d4	Pos	104	69	26	14	HMDB00112	119	C00334
Gluconolactone	1.42	Glucose-d7	Neg	177	129	20	8	HMDB00150	7027	C00198
Glucosamine-6-phosphate	1.04	Glucose-d7	Pos	260	126	20	12	HMDB01254	439217	C00352
Glucose-6-phosphate	2.8	Glucose-d7	Neg	259	139	27	16	HMDB01401	5958	C00092

Glutaric acid	3.02	Alanine-d4	Neg	131	69	30	14	HMDB00661	743	C00489
Glutathione	1.9	L-Citrulline-d7	Pos	308	179	18	12	HMDB00125	124886	C00051
Glyceric acid	1.33	Alanine-d4	Pos	251	145	25	18	HMDB00139	439194	C00258
Glycerol	1.05	Glucose-d7	Neg	93	57	20	8	HMDB00131	753	C00116
Glycine	0.95	Alanine-d4	Pos	76	30	19	12	HMDB00123	750	C00037
Glycolic acid	2.13	D-Fructose(2-13C)	Pos	77	51	57	14	HMDB00115	757	C00160
Glycoursodeoxycholic acid	6.87	D-Fructose(2-13C)	Neg	448	74	72	36	HMDB00708	12310288	NA
Guanine	0.86	Valine-d8	Pos	152	135	20	20	HMDB00132	764	C00242
Guanosine	1.5	Guanosine-15N5	Neg	282	149.8	42	18	HMDB00133	6802	C00387
Guanosine diphosphate	4.43	Guanosine-15N5	Neg	442	150	52	24	HMDB01201	8977	C00035
Guanosine monophosphate	4.8	Guanosine-15N5	Neg	362	79	28	29	HMDB01397	6804	C00144
Guanosine triphosphate	5.41	Guanosine-15N5	Neg	522	158.83	46	28	HMDB01273	6830	C00044
Homocitrulline	1.05	Ornithine-d6	Pos	190	127	12	16	HMDB00679	65072	C02427
Homo-arginine	3.37	D-Fructose(2-13C)	Pos	189	144	25	15	HMDB00670	9085	C01924
Homovanillic acid	4.34	Alanine-d1	Pos	183	94	18	26	HMDB00118	1738	C05582
Hydroxykynurenine	1.22	Methionine-d3	Neg	223	206	28	7	HMDB00732	89	C02794
Hypoxanthine	1.15	D-Fructose(2-13C)	Neg	135	92	44	18	HMDB00157	790	C00262
Indoleacetic acid	5.86	Methyl malonate-d3	Pos	176	103	34	30	HMDB00197	802	C00954
Inosine	1.41	Inosine-15N4	Neg	267	134.93	44	26	HMDB00195	6021	C00294
Inosine diphosphate	4.39	Inosine-15N4	Neg	427	134.92	50	22	HMDB03335	644173	C00104
Inosine triphosphate	5.41	Inosine-15N4	Neg	508	441.9	62	60	HMDB00189	8583	C00081
Isopentenyl pyrophosphate	4.95	D-Fructose(2-13C)	Neg	245	79	50	25	HMDB01347	1195	C00129
Isoxanthopterin	1.38	D-Fructose(2-13C)	Neg	178	136	14	30	HMDB00704	10729	C03975
Itaconic acid	4.07	D-Fructose(2-13C)	Neg	129	85	22	12	HMDB02092	811	C00490
L-Acetylcarnitine	1.26	Carnitine-d9	Pos	204	85	20	21	HMDB00201	7045767	C02571
L-Alloisoleucine	2	Leucine-d3	Pos	132	69	20	18	HMDB00557	99288	C21096
L-Arginine	0.88	Arginine-d7	Pos	175	70	32	20	HMDB00517	6322	C00062
L-Asparagine	2.01	Arginine-d7	Pos	133	87	26	8	HMDB00168	6267	C00152
L-Carnitine	1.14	Carnitine-d9	Pos	162	85	15	11	HMDB00062	2724480	C15025
L-Cystathionine	0.91	Ornithine-d6	Neg	221	134	30	12	HMDB00099	439258	C02291
L-Cystine	3.37	Ornithine-d6	Pos	241	109	32	20	HMDB00192	67678	C00491
L-Dihydroorotic acid	2.75	Ornithine-d6	Neg	157	114	34	18	HMDB02923	439216	C00337
L-Glutamine	0.84	Glutamate-d5	Pos	147	84	22	16	HMDB00641	5961	C00064
L-Histidine	0.86	Leucine-d3	Pos	83	83	26	22	HMDB00177	6274	C00135
L-Homoserine	1.1	Leucine-d3	Neg	118	100	30	8	HMDB00719	12647	C00263
L-Isoleucine	2.01	Leucine-d3	Pos	132	86	28	14	HMDB00172	6306	C00407
Lithium acetoacetate	1.97	Alanine-d4	Pos	109	65	30	13	HMDB0000060	96	C00164
L-Kynurenine	1.89	D-Fructose(2-13C)	Neg	207	144	25	17	HMDB00684	161166	C00328
L-Lactic acid	2.73	Alanine-d4	Pos	89	43	30	12	HMDB00190	107689	C00186
L-Leucine	2	Leucine-d3	Pos	132	69	26	16	HMDB00687	6106	C00123

L-Lysine	0.88	Ornithine-d6	Pos	147	84	26	26	HMDB00182	5962	C00047
L-Methionine	1.62	Methionine-d3	Pos	150	56	22	16	HMDB00696	6137	C00073
L-Monapterin	1.07	D-Fructose(2-13C)	Neg	252	191.95	34	16	HMDB0000877	440842	NA
L-Phenylalanine	2.85	Phenylalanine-d5	Pos	166	103	24	28	HMDB00159	6140	C00079
L-Proline	0.9	Alanine-d4	Pos	116	71	22	20	HMDB00162	145742	C00148
L-Ribulose	1.03	Alanine-d4	Neg	149	89	20	7	HMDB03371	439204	C00310
L-Serine	0.95	Ornithine-d6	Neg	104	74	27	11	HMDB00187	5951	C00065
L-Sorbose	0.98	Alanine-d4	Neg	179	89	72	8	HMDB01266	441484	C08356
L-threonine	0.98	Tyrosine-d4	Pos	120	74	24	16	HMDB00167	6288	C00188
L-Tyrosine	2.05	Tyrosine-d4	Pos	182	91	24	26	HMDB00158	6057	C00082
L-Valine	1.36	Valine-d8	Pos	118	72	24	18	HMDB00883	6287	C00183
Malic acid	4	Methyl malonate-d3	Neg	133	71	24	14	HMDB00744	525	C00711
Malonic acid	3.89	Methyl malonate-d3	Neg	103	59	20	10	HMDB00691	867	C00383
Mannitol	0.93	Glucose-d7	Neg	181	89	30	12	HMDB00765	6251	C00392
Melibiose	0.91	Glucose-d7	Neg	341	179	56	12	HMDB00048	440658	C05402
Mevalonic acid-5-phosphate	5.27	Adenosine C13	Pos	227	97	26	16	HMDB01343	439400	NA
Myoinositol	2.36	Glucose-d7	Pos	181	109	18	10	HMDB00211	892	C00137
N-Acetylspermine	0.95	Glucose-d7	Pos	245	99	25	22	HMDB01186	916	C02567
N-Acetyl-D-glucosamine	0.95	Glucose-d7	Neg	220	119	28	10	HMDB00215	439174	C00140
N-Acetyl-D-glucosamine-6-phosphate	2.99	Glucose-d7	Neg	300	97	20	20	HMDB01062	440996	C00357
N-Acetylmannosamine	1.03	Glucose-d7	Pos	221	126	24	17	HMDB01129	11096158	C00645
N-Acetylneuraminic acid (NANA, Sialic acid)	2.63	D-Fructose(2-13C)	Neg	308	87	22	8	HMDB00230	445063	C19910
N-Acetylputrescine	3.79	D-Fructose(2-13C)	Pos	131	72	30	13	HMDB02064	122356	C02714
NADH (Coenzyme I)	4.48	Adenosine C13	Neg	664	78.9	64	62	HMDB01487	439153	C00004
NADP (Coenzyme II)	4.4	Adenosine C13	Neg	742	619.9	28	18	HMDB00217	5886	C00006
Neopterin	1.07	D-Fructose(2-13C)	Neg	252	192	30	15	HMDB00845	4455	C05926
Niacinamide	2.39	Ornithine-d6	Pos	124	81	6	20	HMDB01406	936	C00153
Nicotinamide ribotide	6.3	Ornithine-d6	Pos	335	123	30	22	HMDB00229	14180	C00455
Nicotinic acid	6.29	Ornithine-d6	Neg	122	78	25	15	HMDB01488	938	C00253
Nicotinic acid mononucleotide	6.26	Ornithine-d6	Pos	336	125	42	22	HMDB01132	53477721	C01185
O-phosphoethanolamine	1.97	Alanine-d4	Pos	142	81	26	20	HMDB00224	1015	C00346
Ornithine	0.9	Ornithine-d6	Pos	134	71	18	14	HMDB00214	6262	C00077
Orotic acid	3.02	Ornithine-d6	Neg	155	111	2	14	HMDB00226	967	C00295
Orotidine-5'-monophosphate	4.41	Ornithine-d6	Neg	367	79	14	14	HMDB00218	160617	C01103
O-Succinyl-L-homoserine	0.96	Alanine-d4	Neg	218	117	30	11	NA	439406	C01118
Oxalacetic acid	1.2	D-Fructose(2-13C)	Neg	133	23	170	20	HMDB00223	970	C00036
Oxalic acid	6.13	Citric acid-d4	Neg	89	61	18	4	HMDB02329	971	C00209
Oxidized glutathione	2.44	Citric acid-d4	Pos	611	306	52	26	HMDB03337	975	C00127

Oxoglutaric acid	0.81	Citric acid-d4	Pos	147	84	25	15	HMDB00208	51	C00026
Pantothenic acid	3.07	Glucose-d7	Pos	239	128	30	20	HMDB00210	988	C00864
Phenylpyruvic acid	4.7	Glucose-d7	Neg	163	91	20	15	HMDB00205	997	C00166
Phosphoserine	5.9	Ornithine-d6	Neg	186	88	44	26	HMDB00272	68841	C01005
Pipecolic acid	1.77	D-Fructose(2-13C)	Pos	130	84	10	10	HMDB00070	849	C00408
Porphobilinogen	2.3	D-Fructose(2-13C)	Neg	226	26	170	52	HMDB00245	1021	C00931
Progesterone	9.63	Phenylalanine-d5	Pos	315	109	44	30	HMDB01830	5994	C00410
Pterin	1.38	D-Fructose(2-13C)	Neg	162	119	30	15	HMDB00802	73000	C00715
Putrescine	0.73	D-Fructose(2-13C)	Pos	89	72	45	9	HMDB01414	1045	C00134
Pyridoxal Hydrochloride	4.04	D-Fructose(2-13C)	Neg	203	167	20	15	HMDB0000239	6171	C00314
Pyridoxal-5'-phosphate	4	D-Fructose(2-13C)	Neg	246	97	27	10	HMDB01491	1051	C00018
Raffinose	1.07	Methionine-d3	Pos	505	163	25	20	HMDB03213	10542	C00492
Ribitol	0.96	Glucose-d7	Neg	151	89	24	10	HMDB00508	NA	C00474
Saccharopine	1.07	Alanine-d4	Pos	277	84	111	20	HMDB00279	160556	C00762
Sarcosine	0.97	Alanine-d4	Pos	90	62.8	26	6	HMDB00271	1088	C00213
Sedoheptulose-7-phosphate	2.72	D-Fructose(2-13C)	Neg	289	97	50	12	HMDB0001068	165007	C05382
Sepiapterin	2.35	D-Fructose(2-13C)	Neg	236	163.99	44	18	HMDB00238	65253	C00835
Sodium 4-methyl-2-oxovalerate	0.89	Alanine-d4	Pos	153	23	27	7	HMDB0000695	70	NA
Sodium glycodeoxycholate	10.11	Glucose-d7	Pos	472.2	397	30	30	HMDB32596	755	C05464
Sodium β-hydroxy isobutyrate	5.39	D-Fructose(2-13C)	Pos	127	84	30	12	HMDB0000336	87	C01188
Sorbitol	1.02	D-Fructose(2-13C)	Neg	181	101	32	10	HMDB00247	5780	C00794
Spermine	0.73	Alanine-d4	Pos	203	129	20	18	HMDB01256	1103	C00750
Sphingosine	9.58	Alanine-d4	Pos	299	252	12	15	HMDB00252	5353955	C00319
S-Sulfocysteine	2.78	D-Fructose(2-13C)	Neg	200	81	25	16	HMDB00731	115015	C05824
Succinic acid	4.05	D-Fructose(2-13C)	Neg	117	73	30	15	HMDB00254	1110	C00042
Taurine	0.95	Ornithine-d6	Neg	124	80	30	18	HMDB00251	1123	C00245
Taurodeoxycholic acid	9.48	Glucose-d7	Pos	522	147	46	28	HMDB00896	2733768	C05463
Thiamine	3.16	Methionine-d3	Pos	266	77	40	18	HMDB00235	1130	C00378
Thymidine	2.1	D-Fructose(2-13C)	Neg	241	42	30	20	HMDB00273	5789	C00214
Thymine	1.46	Adenosine C13	Neg	125	42	26	14	HMDB00262	1135	C00178
Tryptophanol	1.3	Adenosine C13	Neg	189	129	30	15	HMDB03447	10685	C00955
Uracil	3.01	Adenosine C13	Neg	111	42	30	15	HMDB00300	1174	C00106
Ureidopropionic acid	2.53	Adenosine C13	Neg	131	88	18	16	HMDB00026	111	C02642
Uridine	1.28	Adenosine C13	Neg	243	110	36	14	HMDB00296	6029	C00299
Uridine diphosphate-N-acetylglucosamine	4.23	Adenosine C13	Neg	606	282	30	28	HMDB00290	445675	C00043
Uridine-5′-diphospho galactose	2.18	Adenosine C13	Pos	611	449	100	20	HMDB42037	18058	NA
Uridine-5'-diphosphate	4.39	Adenosine C13	Neg	403	158.8	44	28	HMDB00295	6031	C00015
Uridine-5'-mono phosphate	3.1	Adenosine C13	Neg	323	96.83	40	22	HMDB00288	6030	C00105

Uridine-diphospo-glucuronic acid	5.34	Adenosine C13	Neg	579	403	170	25	HMDB00935	17473	C00167
Xanthine	1.23	Adenosine C13	Neg	151	79.87	40	20	HMDB00292	1188	C00385
β-Nicotinamide adenine dinucleotide (NAD)	2.83	Adenosine C13	Neg	662	539	26	16	HMDB0000902	5893	C00003
*2-Deoxyadenosine-C13	2.6	NA	Pos	271	154	150	15	NA	NA	NA
*Adenosine C13	2.31	NA	Neg	267	134	14	14	NA	NA	NA
*Alanine-d1	0.98	NA	Pos	91	45	28	10	NA	NA	NA
*Alanine-d4	0.95	NA	Pos	94	78	18	6	NA	NA	NA
*Arginine-d7	0.91	NA	Pos	182	77	32	20	NA	NA	NA
*Aspartate-d3	1.03	NA	Pos	137	77	58	18	NA	NA	NA
*C10-Carnitine-d3	8.39	NA	Pos	319	85	18	22	NA	NA	NA
*C12-Carnitine-d3	9.71	NA	Pos	247	85	18	20	NA	NA	NA
*C14-Carnitine-d3	10.89	NA	Pos	375	85	18	24	NA	NA	NA
*C16-Carnitine-d3	10.9	NA	Pos	403	85	18	22	NA	NA	NA
*C18-Carnitine-d3	10.18	NA	Pos	431	85	52	24	NA	NA	NA
*C2-Carnitine-d3	1.26	NA	Pos	207	85	28	18	NA	NA	NA
*C3-Carnitine-d3	2.46	NA	Pos	221	85	30	18	NA	NA	NA
*C4-Carnitine-d3	3.1	NA	Pos	235	85	28	20	NA	NA	NA
*C5-Carnitine-d9	3.86	NA	Pos	255	85	28	18	NA	NA	NA
*C6-Carnitine-d3	4.93	NA	Pos	263	85	34	20	NA	NA	NA
*C8-Carnitine-d3	6.8	NA	Pos	291	85	16	20	NA	NA	NA
*Carnitine-d9	2.78	NA	Pos	171	106	24	24	NA	NA	NA
*Citric acid-d4	4.51	NA	Neg	195	132	24	12	NA	NA	NA
*Citrulline-d2	1.03	NA	Pos	178	72	22	20	NA	NA	NA
*D-Fructose(2-13C)	0.99	NA	Neg	180	90	25	10	NA	NA	NA
*Glucose-d7	1.04	NA	Neg	186	124	50	15	NA	NA	NA
*Glutamate-d5	0.99	NA	Pos	153	88	22	14	NA	NA	NA
*Guanosine-15N5	1.48	NA	Neg	155	137	50	15	NA	NA	NA
*Inosine-15N4	1.35	NA	Neg	271	139	30	25	NA	NA	NA
*L-Citrulline-d7	1.04	NA	Neg	181	138	30	15	NA	NA	NA
*Leucine-d3	1.92	NA	Pos	135	89	22	24	NA	NA	NA
*Methionine-d3	0.99	NA	Pos	153	88	22	16	NA	NA	NA
*Methyl malonate-d3	1.07	NA	Pos	120	76	30	8	NA	NA	NA
*Ornithine-d6	0.83	NA	Pos	139	76	18	16	NA	NA	NA
*Phenylalanine-d5	2.82	NA	Pos	171	103	22	26	NA	NA	NA
*Tyrosine-d4	5.33	NA	Pos	186	57	76	24	NA	NA	NA
*Valine-d8	1.07	NA	Pos	126	62	14	20	NA	NA	NA

(Abbrevations: Internal Standard, Neg-Negative, Pos-Positive)

Table A1.3: Metabolites Linearity and lower limit of quantification

Compound ID	Linearity			LLOQ nM
	Intercept	Slope	R^2	
2,3-Pyridinedicarboxylic acid	127.69	1.43	0.993	5
21-Deoxycortisol	22.62	32.587	0.995	750
2-Hydroxyglutaric acid	-68.22	12.65	0.996	750
2-Isopropylmalic acid	-19.92	4.259	0.996	5
2-Ketobutyric acid	49.67	0.859	0.996	1
2-Phosphoglyceric acid	29.36	0.147	0.995	25
3,4-Dihydroxyphenylacetic acid	-45.61	18.622	0.991	25
3-Hydroxyanthranilic acid	35.88	6.877	0.994	750
3-Methylglutaric acid	48.22	0.926	0.991	5
3-Methylhistidine	420.12	193.03	0.995	1
3-Phosphoglyceric acid	0	29.06	0.995	750
4,6-Dioxoheptanoic acid	14.319	12.16	0.992	5
4-Hydroxy-L-glutamic acid	0	28.74	0.995	1
4-Hydroxyphenylpyruvic acid	0	0.822	0.991	750
4-Hydroxyproline	52.34	28.65	0.995	25
5,6-Dihydro-5-methyluracil	0	9.547	0.994	750
5,6-Dihydrouridine	68.75	42.082	0.991	500
5-Aminolevulinic acid	0	0.742	0.992	250
5-Hydroxyindoleacetic acid	0	0.404	0.993	25
5-Hydroxylysine	134.22	440.39	0.997	1
6-Phosphogluconic acid	-120.33	4.666	0.995	5
Acetylcarnitine	-162.33	33.15	0.995	750
Acetyl-CoA	0	0.544	0.992	750
Acetylspermine	12.35	1.36	0.995	1
Adenine	-8.053	0.619	0.996	10
Adenosine	-0.6814	0.102	0.995	5
Adenosine diphosphate	262.44	0.57	0.996	1
Adenosine monophosphate	0.488	0.201	0.992	10
Adenosine triphosphate	-1.846	3.74	0.987	25
Adenylsuccinic acid	-2.572	0.197	0.995	5
Adonitol	0	0.625	0.992	1
Alanine	52.65	27.48	0.997	1
Aldosterone	13.66	0.855	0.992	1
Aminoadipic acid	53.976	4.428	0.991	1
Arabinose	16.04	1.285	0.992	1
Argininosuccinic acid	293.27	0.182	0.996	1
Betaine	62.33	38.77	0.994	1
Biopterin	19.329	1.107	0.996	1
Biotin	72.55	26.56	0.998	500
Cholic acid	82.22	0.284	0.998	750
Citrulline	12.32	80.04	0.998	1

Coenzyme A	-92.33	6.48	0.991	750
Corticosterone	62.22	7.39	0.991	1
Cortisone	40.55	34.74	0.996	5
Creatinine	28.33	60.1	0.992	25
Cyclic AMP	23.45	0.11	0.995	10
Cyclic GMP	28.62	0.29	0.998	100
Cysteine-S-sulfate	-30.55	3.168	0.995	1
Cytidine	-11.07	0.253	0.996	50
Cytidine monophosphate	-19.75	1.14	0.995	25
Cytidine monophosphate-N-acetylneuraminic acid	62.22	1.14	0.995	750
Cytidine triphosphate	-67.05	7.17	0.993	1
Cytosine	26.65	4.37	0.995	1
Cytosine diphosphate	18.12	3.76	0.991	1
D-Arabitol	16.04	1.0002	0.99	1
D-Aspartic acid	293.27	7.934	0.995	1
Deoxyadenosine	102.36	0.196	0.996	1
Deoxyadenosine monophosphate	125.35	0.246	0.988	750
Deoxycytidine	162.35	0.246	0.996	1
Deoxyguanosine	55.26	0.684	0.996	750
Deoxyinosine	75.22	0.148	0.994	5
Deoxyribose-5-phosphate	162.14	0.244	0.995	10
Deoxyuridine	62.48	1.688	0.995	500
D-Erythrose-4-phosphate	-14.54	0.652	0.996	25
Dexamethasone	0	26.54	0.992	1
Dextrose	0	1.668	0.992	1
D-Fructose	0	0.181	0.991	10
D-Glucose	52.77	0.578	0.996	750
D-Glucuronic acid	-4.053	0.197	0.992	1
D-Glutamic acid	104.22	235.47	0.991	1
Dihydrouracil	0	2.465	0.995	750
Dihydroxyacetone phosphate	-2.29	1.028	0.996	5
Dimethylallylpyrophosphate (DMAPP)	88.25	0.216	0.995	5
DL-Homocystine	25.55	94.23	0.993	1
Dopamine	35.87	1.449	0.99	5
D-Threitol	65.01	16.23	0.992	1
D-Tryptophan	68.47	204.06	0.996	750
Dulcitol	24.68	0.682	0.995	1
D-Xylitol	68.22	4.98	0.993	1
Estradiol	104.22	1.41	0.991	1
Estriol	509.18	1.48	0.991	1
Estrone	62.85	5.306	0.992	1
Ethanolamine	106.28	17.09	0.995	1
Fructose-1,6-bisphosphate	10.32	0.525	0.986	1
Fructose-6-phosphate	23.02	2.248	0.991	25

Fumaric acid	76.67	0.112	0.992	25
Galactose-1-phosphate	112.69	2.412	0.993	25
Gamma-Amino Butyric acid (GABA)	394.77	7.02	0.995	1
Gluconolactone	10.554	0.264	0.991	25
Glucosamine-6-phosphate	0	25.86	0.991	25
Glucose-6-phosphate	45.05	2.409	0.994	1
Glutaric acid	0	0.114	0.994	25
Glutathione	68.11	0.855	0.994	750
Glyceric acid	108.22	6.847	0.995	25
Glycerol	107.69	82.56	0.996	25
Glycine	104.57	7.7	0.996	1
Glycolic acid	807.06	28.17	0.994	750
Glycoursodeoxycholic acid	0	38.62	0.992	5
Guanine	196.52	12.74	0.986	5
Guanosine	1.524	0.47	0.994	750
Guanosine diphosphate	11.61	0.318	0.995	1
Guanosine monophosphate	11.69	1.24	0.993	1
Guanosine triphosphate	22.62	0.307	0.993	1
Homo-arginine	63.66	2.62	0.991	5
Homocitrulline	0	45.62	0.99	1
Homovanillic acid	92.77	48.22	0.991	1
Hydroxykynurenine	65.55	0.442	0.991	25
Hypoxanthine	-5.242	0.399	0.995	1
Indoleacetic acid	12.58	52.664	0.991	5
Inosine	-14.27	0.726	0.997	5
Inosine diphosphate	11.07	0.59	0.997	1
Inosine triphosphate	0	0.133	0.991	500
Isopentenyl pyrophosphate	-100.24	28.06	0.995	1
Isoxanthopterin	68.49	0.832	0.995	1
Itaconic (3-methylsuccinic) acid	114.22	23.62	0.992	1
L-Alloisoleucine	128.66	75.06	0.991	750
L-Arginine	163.44	276.08	0.995	1
L-Asparagine	133.9	253.36	0.996	1
L-Carnitine	50.72	10.65	0.993	1
L-Cystathionine	23.444	0.84	0.995	1
L-Cystine	72.94	0.303	0.997	1
L-Dihydroorotic acid	0	1.968	0.993	500
L-Galactono-1,4-lactone	0	5.544	0.996	1
L-Glutamine	274.06	12.409	0.989	1
L-Histidine	152.73	102.69	0.995	1
L-Homoserine	47.68	11.822	0.998	1
L-Isoleucine	162.22	1798	0.995	1
Lithium acetoacetate	89.26	0.745	0.995	25
L-Kynurenine	46.58	0.214	0.995	25

L-Lactic acid	138.22	0.826	0.992	750
L-Leucine	132.66	200.56	0.995	1
L-Lysine	404.3	138.003	0.994	1
L-Methionine	130.22	353.88	0.997	1
L-Phenylalanine	102.33	155.1	0.995	1
L-Proline	0	33.65	0.995	750
L-Ribulose	74.44	1.852	0.997	750
L-Serine	27.98	2.01	0.996	1
L-Sorbose	52.55	0.122	0.994	1
L-Threonine	125.9	44.43	0.993	1
L-Tyrosine	152.3	335.44	0.992	1
L-Valine	182.6	471.002	0.995	1
Malic acid	0	0.249	0.993	750
Malonic acid	296.366	2.208	0.992	1
Maltose	182.55	0.266	0.997	1
Mannitol	162.44	1.586	0.992	750
Mannose	0	3.65	0.992	250
Mannose-1-phosphate	-5.391	0.622	0.996	1
Melibiose	92.66	2.46	0.997	25
Methylmalonic acid	102.44	6.844	0.998	1
Mevalonic acid 3-phosphate	0	56.242	0.996	1
Mevalonic acid-5P	0	3.648	0.992	1
Monapterin	-8.017	1.06	0.996	5
Myoinositol	-18.25	0.284	0.996	1
N-Acetyl-D-glucosamine	-193.77	0.1109	0.996	5
N-Acetyl-D-glucosamine-6-phosphate	-120.66	0.956	0.994	750
N-Acetylmannosamine	0	52.66	0.999	500
N-Acetylneuraminic acid (NANA, Sialic acid)	282.36	2.33	0.993	25
N-Acetylputrescine	62.33	152.6	0.998	1
NADH	-13.061	0.104	0.995	10
NADP	1.733	0.891	0.997	25
Neopterin	-0.739	1.012	0.995	1
Niacinamide	152.56	12.655	0.995	750
Nicotinamide adenine dinucleotide(NAD)	0	0.065	0.996	500
Nicotinamide ribotide	418.45	32.087	0.991	25
Nicotinic acid	22.37	1.182	0.992	750
Nicotinic acid mononucleotide	22.299	28.066	0.996	25
O-Phosphoethanolamine	-151.22	4.15	0.993	750
Ornithine	536.74	25.97	0.993	1
Orotic acid	248.35	1.295	0.994	1
Orotidine-5'-monophosphate	0	1.855	0.992	750
O-Succinyl-L-homoserine	188.2	4.855	0.995	25
Oxalacetic acid	162.44	0.283	0.996	750
Oxalic acid	111.4	5.62	0.996	250

Oxidized glutathione	90.5	0.543	0.994	750
Oxoglutaric acid	56.77	0.648	0.996	750
Pantothenic acid	62.77	64.05	0.996	500
Phenylpyruvic acid	103.22	5.844	0.986	750
Phosphoenolpyruvic acid	-21.919	0.14	0.993	25
Phosphoserine	82.77	12.082	0.997	25
Pipecolic acid	-26.94	0.088	0.993	1
Porphobilinogen	68.48	0.454	0.995	750
Progesterone	46.55	23.12	0.995	5
Pterin	-29.96	1.434	0.997	1
Putrescine	123.61	9.221	0.99	25
Pyridoxal Hydrochloride	-1.318	26.12	0.994	5
Pyridoxal-5'-phosphate	0	0.576	0.992	5
Raffinose	82.44	1.285	0.998	25
Ribitol	75.55	8.64	0.992	250
Ribose-5-phosphate	132.44	0.511	0.995	5
Saccharopine	-18.26	222.08	0.995	750
Sarcosine	62.87	8.655	0.996	750
Sedoheptulose-7-phosphate	82.55	1.74	0.995	5
Sepiapterin	112.5	0.872	0.993	25
Sodium 4-methyl-2-oxovalerate	0	0	0.999	750
Sodium glycodeoxycholate	18.62	33.64	0.996	25
Sodium β-hydroxyisobutyrate	0	66.5	0.998	750
Sorbitol	12.82	0.848	0.991	5
Spermine	45.514	4.11	0.992	25
Sphingosine	62.88	8.062	0.995	750
S-Sulfocysteine	-22.96	2.42	0.996	1
Succinic acid	0	25.62	0.992	750
Taurine	67.82	3.79	0.996	1
Taurodeoxycholic acid	-18.22	62.08	0.995	750
Tauroursodeoxycholic acid	56.78	0.845	0.996	1
Thiamine	-1.734	0.181	0.994	1
Thymidine	13.507	0.326	0.992	1
Thymine	-18.68	0.413	0.995	25
Tryptophanol	-59.22	32.082	0.991	750
Uracil	-1.824	1.59	0.995	25
Ureidopropionic acid	142.55	0.395	0.993	25
Uridine	1.381	0.48	0.995	5
Uridine diphosphate-N-acetylglucosamine	0	5.82	0.996	750
Uridine diphospho-glucuronic acid	-13.551	0.457	0.992	1
Uridine-5′-diphosphogalactose	92.69	0.955	0.996	750
Uridine-5'-diphosphate	-1.882	1.41	0.995	1
Uridine-5'-monophosphate	-4.728	1.386	0.996	5
Xanthine	-130.93	0.114	0.994	100

Table A1.4: Metabolites intraday and interday precision and accuracy

Name	Intra-day precision (%) n=6			Intra-day accuracy (%) n=6			Inter-day precision (%) n=18			Inter-day accuracy (%) n=18		
	750	250	25	750	250	25	750	250	25	750	250	25
α-Hydroxypregnenolone	12.5	11.2	8.2	115	96.5	118.8	14.6	14.3	9.2	105.4	95.7	102.2
17 α-Hydroxyprogesterone	2.8	9.5	11.2	87.5	115.7	96.2	3.8	11.2	10.6	96.5	102.3	118.3
2,3-Pyridinedicarboxylic acid	0.8	13.4	17.8	101	86.6	113.2	1.3	11.0	16.5	103.9	89.0	106.5
21-Deoxycortisol	8.2	BLLOQ	BLLOQ	115	BLLOQ	BLLOQ	10.5	BLLOQ	BLLOQ	85.2	BLLOQ	BLLOQ
2-Hydroxyglutaric acid	10.2	BLLOQ	BLLOQ	86.2	BLLOQ	BLLOQ	12.2	BLLOQ	BLLOQ	106.2	BLLOQ	BLLOQ
2-Isopropylmalic acid	0.6	15.5	6.4	101	87.5	93.6	0.9	18.9	8.8	97.0	91.1	108.8
2-Ketobutyric acid	5.8	15.9	20.9	94.2	110.9	120.2	3.3	14.5	17.5	106.7	118.5	113.5
2-Oxoglutaric acid	18.2	BLLOQ	BLLOQ	89.2	BLLOQ	BLLOQ	11.2	BLLOQ	BLLOQ	104.3	BLLOQ	BLLOQ
2-Phosphoglyceric acid	2.6	0.5	7.2	98.2	115.5	105.2	3.9	0.9	9.1	105.9	112.9	107.1
3,4-Dihydroxyphenylacetic acid	8.2	18.2	10.2	91.5	115.2	98.5	10.5	14.7	12.3	87.5	105.3	92.3
3-Hydroxyanthranilic acid	15.3	BLLOQ	BLLOQ	109	BLLOQ	BLLOQ	12.5	BLLOQ	BLLOQ	118.5	BLLOQ	BLLOQ
3-Methylglutaric acid	11.6	9.8	20.3	103	119.2	86.4	8.3	15.4	17.7	120.7	94.8	97.7
3-Methylhistidine	18	20.2	13.4	112	102.2	86.6	19.1	19.3	18.2	108.9	106.7	91.8
3-Phosphoglyceric acid	5.3	BLLOQ	BLLOQ	103	BLLOQ	BLLOQ	4.9	BLLOQ	BLLOQ	115.1	BLLOQ	BLLOQ
4,6-Dioxoheptanoic acid	0.8	12.6	3.4	99.2	107.4	96.6	1.4	14.9	5.4	95.6	118.1	95.4
4-Hydroxy-L-glutamic acid	13.8	13.7	2.8	86.2	116.3	86.8	14.9	19.3	3.2	95.1	118.7	112.8
4-Hydroxyphenylpyruvic acid	16.2	BLLOQ	BLLOQ	92.5	BLLOQ	BLLOQ	19.0	BLLOQ	BLLOQ	102.5	BLLOQ	BLLOQ
4-Hydroxyproline	4.9	14.7	1.6	95.1	114.7	99.4	4.2	18.5	1.2	104.2	91.5	89.2
5,6-Dihydro-5-methyluracil	5.6	BLLOQ	BLLOQ	89.2	BLLOQ	BLLOQ	5.3	BLLOQ	BLLOQ	99.2	BLLOQ	BLLOQ
5,6-Dihydrouridine	17.2	11.2	5.8	93.6	109.6	94.6	20.2	16.5	7.8	112.3	111.3	100.6
5-Aminolevulinic acid	12.6	2.6	BLLOQ	116	105	BLLOQ	11.3	3.2	BLLOQ	88.7	115.8	BLLOQ
5-Hydroxyindoleacetic acid	8.2	BLLOQ	BLLOQ	112	BLLOQ	BLLOQ	7.8	BLLOQ	BLLOQ	118.2	BLLOQ	BLLOQ
5-Hydroxylysine	12.8	20.1	15.1	87.2	102.1	88.9	14.4	17.5	19.3	88.6	92.5	93.7
6-Phosphogluconic acid	4.8	15.2	16.6	119	98.2	119.6	6.2	17.3	18.9	107.5	118.4	103.2
Acetyl-CoA	5.2	BLLOQ	BLLOQ	92.8	BLLOQ	BLLOQ	8.4	BLLOQ	BLLOQ	86.5	BLLOQ	BLLOQ
Adenine	6.5	10.2	4.2	85.6	102	98.2	5.5	12.5	6.2	94.5	117.5	103.8
Adenosine	14.1	12.1	4.1	103	103.7	119	19.3	15.2	5.5	90.7	120.2	100.5
Adenosine diphosphate	16.5	17.4	1.7	104	117.4	105.7	14.2	18.5	3.2	94.2	101.5	109.2
Adenosine monophosphate	11	4.6	15.4	120	95.4	115.4	10.3	6.9	19.4	110.3	106.9	119.4
Adenosine triphosphate	3.4	7.6	17.4	103	93	97.4	4.8	9.2	16.5	118.7	105.8	116.4

Adenylsuccinic acid	12.5	13	20.1	108	113	104.1	11.4	19.6	23.6	118.6	110.4	103.6
Adonitol	2.8	8.5	5.6	98.2	102.8	93.1	3.2	9.8	6.6	108.2	105.3	99.1
Alanine	5.7	12.4	10.5	106	112.4	120.5	7.3	11.6	9.9	116.7	120.6	120.1
Aldosterone	16.5	6.2	7.2	94.5	85.2	118.5	17.6	8.8	6.5	102.4	91.2	113.5
Aminoadipic acid	3.1	3.2	12.8	96.9	96.8	112.8	4.1	5.0	16.8	93.9	90.0	117.2
Arabinose	9.2	6.8	5.7	98.5	112.5	88.5	11.0	7.9	7.5	102.0	107.9	120.5
Argininosuccinic acid	12.5	13	9.1	118	113	94.1	17.6	11.5	17.7	112.4	108.5	105.3
Betaine	6.5	16.7	12	107	116.7	88	8.1	16.4	10.1	116.9	107.6	99.9
Biopterin	6.4	18.3	17.5	106	111.7	112.5	8.8	19.3	18.2	101.8	102.7	99.8
Biotin	16.8	4.8	BLLOQ	95.2	88.5	BLLOQ	13.1	3.4	BLLOQ	105.6	85.9	BLLOQ
Cholic acid	16.5	BLLOQ	BLLOQ	97.2	BLLOQ	BLLOQ	18.8	BLLOQ	BLLOQ	87.2	BLLOQ	BLLOQ
Citrulline	13.2	16.6	11.1	103	103.4	98.9	18.2	15.3	12.9	108.2	95.3	88.9
Coenzyme A	2.7	BLLOQ	BLLOQ	111	BLLOQ	BLLOQ	3.8	BLLOQ	BLLOQ	120.2	BLLOQ	BLLOQ
Corticosterone	17.1	14.7	15.2	103	88.3	114.8	20.8	18.2	18.3	117.2	98.8	104.7
Cortisone	10.2	11.8	12.9	89.2	91.8	94.9	11.4	14.9	18.8	93.6	102.1	96.2
Creatinine	12.3	15.4	15.3	87.7	94.6	85.3	15.8	16.6	18.6	94.2	103.4	102.6
Cyclic AMP	17.9	15.7	12	88.5	102.5	101.2	19.5	18.2	14.6	87.5	95.8	117.4
Cyclic GMP	5.2	9.6	13	94.8	89.4	113	8.8	11.6	18.3	108.8	111.6	108.3
Cysteine-S-sulfate	2.1	10.5	11.9	97.9	89.5	88.1	3.3	15.4	13.6	92.7	105.4	103.6
Cytidine	10.2	15	11	89.8	95	111	12.5	14.4	13.0	109.5	115.6	120.0
Cytidine monophosphate	9.6	6.2	8.6	108	103.8	108.6	10.8	8.5	12.2	95.2	105.5	112.2
Cytidine monophosphate N-acetylneuraminic acid	5.2	BLLOQ	BLLOQ	115	BLLOQ	BLLOQ	9.9	BLLOQ	BLLOQ	120.2	BLLOQ	BLLOQ
Cytidine triphosphate	2.2	4.1	11	97.8	95.9	109	3.3	6.8	13.3	101.7	117.2	120.3
Cytosine	19.3	12.4	13.3	119	85.4	93.3	20.0	11.2	15.1	120.0	98.8	104.9
Cytosine diphosphate	7.6	6.8	6.5	108	93.2	103.5	9.6	10.8	8.0	112.4	87.2	92.0
D-Arabitol	15.8	10.2	16.8	94.2	119.8	106.8	18.2	11.9	19.3	113.2	88.1	109.3
D-Aspartic acid	8.2	11.1	10	91.8	108.9	90	11.5	17.9	14.7	108.5	112.1	114.7
D-Cytosine monophosphate	7.6	16.2	BLLOQ	108	113.8	BLLOQ	8.0	11.4	BLLOQ	120.3	103.2	BLLOQ
Deoxyadenosine	13	13.4	16	92.7	113.5	96.8	18.0	14.6	15.3	112.0	105.4	108.7
Deoxyadenosine monophosphate	8.5	BLLOQ	BLLOQ	86.5	BLLOQ	BLLOQ	13.0	BLLOQ	BLLOQ	102.3	BLLOQ	BLLOQ
Deoxycytidine	8.6	20.1	6.5	91.4	120.1	106.5	9.9	18.5	9.2	100.1	116.5	119.2
Deoxyguanosine	12.8	BLLOQ	BLLOQ	85.5	BLLOQ	BLLOQ	17.0	BLLOQ	BLLOQ	85.5	BLLOQ	BLLOQ
Deoxyinosine	14.1	12.1	4.1	85.9	85.9	120.1	17.8	11.2	10.5	92.2	88.8	112.5
Deoxyribose-5-phosphate	13.9	18.1	13.5	106	111.9	86.5	18.8	20.1	17.3	88.8	97.1	87.3
Deoxyribose-5-phosphate	13.9	18.1	13.5	116	111.9	96.5	12.3	20.1	15.6	119.3	115.2	100.3
Deoxyuridine	4.8	BLLOQ	BLLOQ	113	BLLOQ	BLLOQ	7.2	BLLOQ	BLLOQ	120.5	BLLOQ	BLLOQ
D-Erythrose-4-	1.2	16	6.4	101	114	106.	2.5	18.3	8.4	119.	112.4	110.6

phosphate						4				6		
Dexamethasone	6.8	4.2	6.2	88.8	99.2	95.5	8.8	5.6	7.2	98.3	105.3	115.6
Dextrose	5.8	16.2	11.2	88.5	119.2	85.2	7.7	18.2	15.3	87.6	120.3	87.3
D-Fructose	8.7	16.2	4.8	109	112	98.2	9.4	17.9	6.5	100.6	102.1	93.5
D-Glucose	16.8	BLLOQ	BLLOQ	106	BLLOQ	BLLOQ	20.3	BLLOQ	BLLOQ	109.6	BLLOQ	BLLOQ
D-Glucuronic acid	15.3	9	7.7	115	86	101.7	19.5	11.3	8.3	116.8	99.2	105.7
D-Glutamic acid	12.5	12.1	3.6	97.5	92.1	103.6	10.3	18.1	4.2	88.6	102.3	114.6
Dihydrouracil	12.8	BLLOQ	BLLOQ	106	BLLOQ	BLLOQ	18.8	BLLOQ	BLLOQ	115.2	BLLOQ	BLLOQ
Dihydroxyacetone phosphate	11.3	4.5	18.2	88.7	104.5	118.2	14.8	5.6	20.3	96.5	113.2	120.4
Dimethylallylpyrophosphate	6.8	9.6	19.6	89.2	108.4	118.4	9.2	10.5	20.8	104.3	118.5	120.4
DL-Homocystine	9.6	14.6	4.8	90.4	104.6	104.8	11.2	15.2	5.6	99.5	98.2	114.8
D-Maltose	16.8	11	20	113	99.2	98.5	19.2	14.3	20.4	118.3	103.0	114.5
D-Mannose	8.2	16.2	BLLOQ	85.4	96.2	BLLOQ	12.4	19.8	BLLOQ	105.4	116.2	BLLOQ
D-Mannose-1-phosphate	12	6.4	6.8	108	93.6	106.8	15.2	7.8	9.2	89.8	113.5	116.9
Dopamine	8.7	10	16.2	91.3	100	96.2	7.2	12.3	18.4	95.3	107.3	111.4
D-Ribose-5-phosphate	6.9	18.1	13.5	96.1	111.9	86.5	9.2	20.1	15.6	112.3	98.6	99.6
D-Threitol	0.3	4.2	18.5	99.7	104.2	115.5	0.4	5.2	20.3	104.5	114.5	119.5
D-Tryptophan	5.8	BLLOQ	BLLOQ	95.8	BLLOQ	BLLOQ	8.2	BLLOQ	BLLOQ	105.8	BLLOQ	BLLOQ
Dulcitol	14.2	5.3	5.2	106	118.2	101.2	17.3	6.8	7.2	116.2	120.3	116.2
D-Xylitol	15.5	13.6	13.1	116	89.6	93.1	18.2	19.2	14.3	117.2	98.5	99.3
D-Xylose	2.8	BLLOQ	BLLOQ	97.2	BLLOQ	BLLOQ	3.2	BLLOQ	BLLOQ	107.2	BLLOQ	BLLOQ
Estradiol	13.1	12.6	14.6	96.9	103.4	104.6	14.8	16.2	18.6	108.6	112.3	120.3
Estriol	5.8	4.2	6.2	88.5	89.2	102.5	7.2	5.8	9.2	91.5	98.5	112.5
Estrone	0.1	0.9	10.6	100	99.1	90.6	0.6	1.2	12.3	105.3	105.2	88.3
Ethanolamine	17.5	18.1	11.4	103	102.9	86.4	20.2	19.3	16.4	116.5	94.2	106.4
Fructose-1,6-bisphosphate	14.3	18.9	1.4	95.7	101.1	98.6	20.3	12.3	1.9	105.5	98.1	103.6
Fructose-6-phosphate	6.5	8.1	12.4	93.5	108.1	87.6	9.9	10.3	15.5	105.3	112.1	109.5
Fumaric acid	8.5	6.8	BLLOQ	120	105.7	BLLOQ	12.3	9.2	BLLOQ	120.3	118.2	BLLOQ
Galactose-1-phosphate	2.2	8.8	12.7	97.8	108.8	107.3	3.5	9.2	14.8	107.2	118.5	120.3
Gamma-aminobutyric acid (GABA)	10.1	17.4	14.6	110	92.6	112.6	18.3	20.3	12.3	88.3	102.4	119.3
Gluconolactone	13.4	11.7	15.7	117	118.3	105.7	16.4	12.4	19.2	118.3	120.5	115.7
Glucosamine-6-phosphate	5.8	18.2	20.2	102	112.2	91.2	7.2	20.2	18.2	114.2	106.9	119.8
Glucose-6-phosphate	2.1	10.9	19.3	97.9	110.9	109.3	2.3	12.9	17.5	115.7	87.1	117.5
Glutaric acid	1.9	12.5	13.5	102	117.5	103.5	1.2	14.8	16.2	111.8	120.3	113.5
Glutathione	12.3	BLLOQ	BLLOQ	96.5	BLLOQ	BLLOQ	13.9	BLLOQ	BLLOQ	95.2	BLLOQ	BLLOQ
Glyceric acid	7.9	14.5	15.9	92.1	85.5	86.1	10.8	10.3	16.5	106.2	110.3	96.5
Glycerol	12.6	6.7	5.4	97.4	116.7	96.4	9.2	10.2	8.4	107.2	120.3	111.3

Glycine	12.2	18.6	17.9	112	118.6	120.1	18.5	14.4	16.4	91.5	112.6	118.4
Glycolic acid	18.2	BLLOQ	BLLOQ	92.3	BLLOQ	BLLOQ	14.4	BLLOQ	BLLOQ	112.3	BLLOQ	BLLOQ
Glycoursodeoxycholic acid	18.2	16.3	19.4	109	95.7	98.1	20.8	19.4	20.7	118.0	109.0	102.7
Guanine	17	12.5	4	103	87.5	96	12.9	18.3	6.0	107.1	97.7	96.0
Guanosine	6.2	BLLOQ	BLLOQ	88.3	BLLOQ	BLLOQ	9.2	BLLOQ	BLLOQ	108.3	BLLOQ	BLLOQ
Guanosine diphosphate	6.2	17.9	19.6	103	93.5	96.8	9.0	19.9	15.5	104.0	91.9	108.5
Guanosine monophosphate	0.2	5.2	8.9	98	103	119	0.6	7.4	12.1	106.0	114.2	113.1
Guanosine triphosphate	17.3	16.5	19.8	93.2	118	120.4	18.7	17.7	17.3	97.3	102.3	117.3
Homocitrulline	6.2	8.2	11.2	97.6	98.4	117.2	8.5	9.1	13.0	101.5	101.9	100.0
Homo-L-arginine	5.1	8.9	13	94.9	91.1	113	2.7	6.9	14.8	87.3	93.1	105.8
Homovanillic acid	13.5	13	9.2	90.5	85.8	107.5	17.0	18.6	7.8	107.0	91.4	115.2
Hydroxykynurenine	11.6	17.2	6.8	102	111.1	98.2	14.2	20.3	8.8	113.2	109.2	100.2
Hypoxanthine	17.4	2.8	7.4	107	102.8	92.6	20.3	3.2	9.9	119.2	98.2	102.4
Indoleacetic acid	5.9	19.2	18.1	102	103.8	92.5	9.1	12.1	16.2	120.1	92.1	106.2
Inosine	11.9	8.8	6.4	102	108.2	116.4	15.4	11.2	8.5	105.4	118.8	103.5
Inosine diphosphate	0.2	11.4	13.5	100	111.4	98.5	0.3	15.2	19.2	112.3	102.3	105.3
Inosine triphosphate	16.4	BLLOQ	BLLOQ	88.6	BLLOQ	BLLOQ	13.7	BLLOQ	BLLOQ	95.4	BLLOQ	BLLOQ
Isopentenyl pyrophosphate	17.9	9	8.3	92.1	109	118.3	20.3	11.3	9.4	103.2	111.4	120.4
Isoxanthopterin	2.5	15.7	13	97.5	115.7	103	4.3	20.9	12.7	110.3	120.9	119.2
Itaconic (3-methylsuccinic) acid	14.2	18	17.2	111	103	112.9	19.2	20.3	19.3	102.3	111.4	120.2
L-Acetylcarnitine	10.8	BLLOQ	BLLOQ	106	BLLOQ	BLLOQ	13.8	BLLOQ	BLLOQ	118.2	BLLOQ	BLLOQ
L-Alloisoleucine	19.2	BLLOQ	BLLOQ	119	BLLOQ	BLLOQ	15.3	BLLOQ	BLLOQ	102.5	BLLOQ	BLLOQ
L-Arginine	2.7	15.9	12.7	97.3	115.9	102.7	3.2	18.2	15.6	107.5	108.2	98.5
L-Asparagine	8.2	1.4	14.1	91.8	101.4	105.9	9.9	2.3	19.3	101.3	111.2	115.4
L-Carnitine	16.1	14.1	6.4	88.9	115.1	86.4	19.1	12.6	9.7	89.9	102.6	102.7
L-Cystathionine	10.5	17.1	13.4	121	90.1	100.4	12.4	19.2	15.2	120.3	98.3	105.2
L-Cystine	14.9	15.4	12.4	115	105.4	112.4	18.2	16.3	11.2	119.2	109.3	120.3
L-Dihydroorotic acid	18.2	BLLOQ	BLLOQ	113	BLLOQ	BLLOQ	20.2	BLLOQ	BLLOQ	119.3	BLLOQ	BLLOQ
L-Galactono-1,4-lactone	12	16.5	5.3	88.2	106.2	112	15.2	19.2	7.2	96.2	119.3	115.3
L-Glutamine	9.7	10.2	17.6	90.3	89.8	102.4	11.2	12.5	19.2	102.3	90.2	92.3
L-Histidine	3.3	15.4	17.3	103	85.4	117.3	5.2	18.2	19.2	113.2	98.2	120.3
L-Homoserine	15	19.2	2.2	98.5	109.3	101	19.2	20.2	3.6	104.2	112.2	104.2
L-Isoleucine	7.4	5.3	10.5	92.6	105.3	89.5	8.4	9.6	16.5	111.6	107.4	93.5
Lithium acetoacetate	14.2	3.5	5.8	104	92.5	92.5	17.9	5.6	8.9	86.1	107.4	100.1
L-Kynurenine	11.9	7.7	15.3	88.1	107.7	111.3	16.9	6.7	19.6	93.1	113.3	119.6
L-Lactic acid	19.2	BLLOQ	BLLOQ	99.3	BLLOQ	BLLOQ	20.3	BLLOQ	BLLOQ	109.3	BLLOQ	BLLOQ
L-Leucine	8.6	11.7	1.5	91.4	111.7	98.5	11.1	17.7	1.9	108.9	118.3	108.5

L-Lysine	2.2	17.3	18.2	97.8	100.7	118.2	3.5	19.6	20.2	110.5	88.4	120.2
L-Methionine	7.3	10.2	17.7	100	90.2	117.7	8.3	15.2	17.7	111.7	88.8	87.7
L-Phenylalanine	8.2	BLLOQ	BLLOQ	91	BLLOQ	BLLOQ	9.2	BLLOQ	BLLOQ	100.2	BLLOQ	BLLOQ
L-Proline	7.7	BLLOQ	BLLOQ	97.7	BLLOQ	BLLOQ	8.3	BLLOQ	BLLOQ	112.3	BLLOQ	BLLOQ
L-Ribulose	7.4	BLLOQ	BLLOQ	104	BLLOQ	BLLOQ	10.4	BLLOQ	BLLOQ	114.6	BLLOQ	BLLOQ
L-Serine	6.1	2.6	19.8	106	102.6	109.8	9.4	3.6	17.1	108.6	107.4	120.1
L-Sorbose	7.5	6.2	9.8	93.8	102.4	118.2	9.5	8.4	11.3	103.4	113.4	100.4
L-Threonine	14.6	11.5	12.3	95.4	101.5	120.3	20.3	16.5	9.6	88.4	111.3	119.4
L-Tyrosine	5	18.8	10.3	95	109.8	90.3	7.5	15.3	8.3	105.3	113.4	89.3
L-Valine	17.4	10.6	9.4	113	110.6	110.6	15.5	11.2	8.3	102.3	103.4	118.5
Malic acid	6.9	BLLOQ	BLLOQ	109	BLLOQ	BLLOQ	5.4	BLLOQ	BLLOQ	99.5	BLLOQ	BLLOQ
Malonic acid	16.5	15.3	7.9	93.5	94.7	107.9	14.3	11.3	5.6	103.2	115.6	111.3
Mannitol	18.2	BLLOQ	BLLOQ	99.2	BLLOQ	BLLOQ	15.2	BLLOQ	BLLOQ	103.4	BLLOQ	BLLOQ
Melibiose	16.8	12.4	14.2	118	112.8	118.5	19.2	11.2	19.3	104.3	114.3	98.3
Methylmalonic acid	6.2	5.8	4.2	108	116.8	98.2	8.5	6.7	3.4	119.5	103.3	86.6
Mevalonic acid 3-phosphate	8.2	1.2	9.2	113	88.5	115.5	11.2	1.4	5.0	118.8	98.6	115.0
Mevalonic acid-5-phosphate	11.2	6.8	5.2	98.7	102	97.2	13.6	7.5	4.6	103.6	108.5	85.4
Monapterin	13.2	16.7	10.3	86.8	103.3	119.7	11.8	14.0	12.4	91.8	94.0	110.4
Myoinositol	11.2	8.2	6.5	91.5	112.8	101.5	17.5	16.0	7.2	112.5	104.0	97.2
N-Acetyl-D-glucosamine	13.2	16.5	2.9	96.8	103.5	97.1	11.8	19.4	5.5	97.2	87.6	105.5
N-Acetyl-D-Glucosamine 6-Phosphate	13.5	BLLOQ	BLLOQ	112	BLLOQ	BLLOQ	9.8	BLLOQ	BLLOQ	116.9	BLLOQ	BLLOQ
N-Acetylmannosamine	8.2	5.2	BLLOQ	99.5	91.5	BLLOQ	12.4	7.9	BLLOQ	92.3	104.2	BLLOQ
N-Acetylneuraminic acid (NANA, Sialic acid)	8.6	8.8	18.5	91.4	111.2	118.5	6.2	7.6	17.6	89.8	100.4	107.6
N-Acetylputrescine	8.5	13.8	5.2	100	111.2	92.5	9.3	11.3	6.4	99.2	119.2	108.3
N-Acetylspermine	6.6	16.1	19.2	93.4	103.9	103.8	5.7	10.6	20.5	88.3	99.4	113.5
NADH	11.3	12.8	20.2	98.7	107.2	111.2	12.7	16.4	17.2	107.3	113.6	117.2
NADP	0.5	7.3	14.9	99.5	117.3	114.9	0.9	9.8	17.7	107.8	120.2	112.3
Neopterin	4.7	14.8	15.5	95.3	115.2	105.5	3.6	17.5	16.9	86.4	112.5	90.1
Niacinamide	6.7	BLLOQ	BLLOQ	96.7	BLLOQ	BLLOQ	4.7	BLLOQ	BLLOQ	88.9	BLLOQ	BLLOQ
Nicotinamide ribotide	15.8	11.4	12.6	94.2	88.6	88.4	17.9	15.6	13.7	104.1	107.6	98.7
Nicotinic acid	6.4	BLLOQ	BLLOQ	109	BLLOQ	BLLOQ	8.5	BLLOQ	BLLOQ	119.4	BLLOQ	BLLOQ
Nicotinic acid mononucleotide	8.7	13.1	16.5	89.2	115.6	92.5	9.9	15.4	20.0	97.7	116.3	104.0
O-Phosphoethanolamine	6.9	BLLOQ	BLLOQ	115	BLLOQ	BLLOQ	8.3	BLLOQ	BLLOQ	108.6	BLLOQ	BLLOQ
Ornithine	2.1	9.9	17.9	97.9	109.9	117.9	3.1	11.2	13.2	107.9	118.8	106.8
Orotic acid	7.8	0.4	18.6	108	99.6	101.4	10.7	0.5	20.4	89.3	103.0	112.6

Orotidine-5'-monophosphate	6.5	4.3	BLLOQ	118	104.2	BLLOQ	7.4	5.6	BLLOQ	112.3	106.6	BLLOQ
O-Succinyl-L-homoserine	16.6	9.2	4.2	103	98.8	119.2	13.4	11.2	5.3	99.4	102.3	109.3
Oxalacetic acid	8.8	BLLOQ	BLLOQ	98.2	BLLOQ	BLLOQ	10.3	BLLOQ	BLLOQ	112.3	BLLOQ	BLLOQ
Oxalic acid	13.8	16.2	BLLOQ	108	107.4	BLLOQ	11.4	20.3	BLLOQ	98.5	118.4	BLLOQ
Oxidized glutathione	18.2	BLLOQ	BLLOQ	99.2	BLLOQ	BLLOQ	20.3	BLLOQ	BLLOQ	112.3	BLLOQ	BLLOQ
Pantothenic acid	16.2	18.2	BLLOQ	86.5	95.4	BLLOQ	10.2	14.7	BLLOQ	89.3	105.7	BLLOQ
Phenylpyruvic acid	16.8	BLLOQ	BLLOQ	108	BLLOQ	BLLOQ	20.3	BLLOQ	BLLOQ	112.3	BLLOQ	BLLOQ
Phosphoenolpyruvic acid	13.2	15.1	12.5	113	105.1	112.5	16.9	13.2	9.2	93.1	111.2	109.2
Phosphoserine	2.8	5.2	BLLOQ	88.8	109	BLLOQ	3.6	7.6	BLLOQ	102.3	111.5	BLLOQ
Pipecolic acid	18.8	19.1	15.5	91.2	119.1	95.5	14.3	12.8	14.3	105.7	97.2	104.3
Porphobilinogen	9.2	BLLOQ	BLLOQ	96.8	BLLOQ	BLLOQ	7.8	BLLOQ	BLLOQ	102.3	BLLOQ	BLLOQ
Progesterone	6.3	20.2	12.9	106	107.8	87.1	5.3	16.7	11.1	94.7	86.3	97.9
Pterin	14.8	19.8	11.6	85.2	91.2	101.6	12.2	20.0	12.6	97.8	98.0	102.6
Putrescine	12.5	2.6	13.1	107	102.6	116.9	17.9	3.0	15.7	112.1	96.0	104.3
Pyridoxal Hydrochloride	12.8	14.6	9.2	108	115	117.2	10.4	11.5	7.6	99.2	112.4	107.5
Pyridoxal-5'-phosphate	15	16.1	11.6	85	113.9	98.4	11.2	17.9	15.8	105.8	102.1	105.8
Raffinose	18.2	7.9	5.6	95.2	91.2	116.5	13.4	9.2	6.2	95.6	89.4	105.7
Ribitol	6.2	19.2	BLLOQ	98.2	111.2	BLLOQ	9.5	12.4	BLLOQ	105.4	103.4	BLLOQ
Saccharopine	18.8	12.3	14.3	91.2	101.7	115.7	19.3	18.5	18.2	100.7	91.5	119.8
Sarcosine	13.2	BLLOQ	BLLOQ	98.2	BLLOQ	BLLOQ	11.2	BLLOQ	BLLOQ	109.2	BLLOQ	BLLOQ
Sedoheptulose-7-phosphate	4.3	14.6	8.2	95.7	95.4	98.2	5.9	15.8	10.6	92.1	104.2	109.4
Sepiapterin	8.2	9.2	18.2	106	118.2	102	10.0	12.3	15.0	118.3	113.7	105.0
Sodium 4-methyl-2-oxovalerate	7.2	BLLOQ	BLLOQ	116	BLLOQ	BLLOQ	9.3	BLLOQ	BLLOQ	98.7	BLLOQ	BLLOQ
Sodium glycodeoxycholate	13.2	9.5	15.8	92.8	92.4	115.2	19.2	11.4	18.6	112.3	99.4	102.9
Sodium β-hydroxyisobutyrate	7.2	BLLOQ	BLLOQ	95.2	BLLOQ	BLLOQ	6.4	BLLOQ	BLLOQ	102.3	BLLOQ	BLLOQ
Sorbitol	16.5	20.3	18.9	106	118.4	104.2	12.3	13.2	20.9	112.3	111.3	97.3
Spermine	19.1	13.8	18.7	109	86.2	111.3	17.3	11.0	20.5	112.7	109.0	109.5
Sphingosine	15.3	BLLOQ	BLLOQ	107	BLLOQ	BLLOQ	20.5	BLLOQ	BLLOQ	114.3	BLLOQ	BLLOQ
S-Sulfocysteine	20.9	13.2	11.5	121	106.8	91.5	12.2	11.1	14.2	119.2	86.9	114.2
Succinic acid	7.2	BLLOQ	BLLOQ	116	BLLOQ	BLLOQ	5.4	BLLOQ	BLLOQ	108.4	BLLOQ	BLLOQ
Taurine	13.3	6.5	17.6	86.7	1000.5	92.4	12.0	8.3	20.8	98.0	108.3	103.2
Taurodeoxycholic acid	2.8	BLLOQ	BLLOQ	98.2	BLLOQ	BLLOQ	1.5	BLLOQ	BLLOQ	104.3	BLLOQ	BLLOQ
Tauroursodeoxycholic Acid	5.2	10.3	16.2	112	85	89.5	7.0	11.5	20.2	91.0	98.5	99.8
Thiamine	0.8	14.1	18.7	101	85.9	99.7	1.2	13.3	14.5	93.8	96.7	104.5
Thymidine	18.9	11.2	14	109	98.8	86	16.3	14.1	16.8	103.7	85.9	113.2
Thymine	9.5	17.4	15.3	90.5	102.6	105.3	5.1	18.3	11.7	104.9	91.7	118.3

Tryptophanol	2.8	BLLOQ	BLLOQ	109	BLLOQ	BLLOQ	3.7	BLLOQ	BLLOQ	98.3	BLLOQ	BLLOQ
Uracil	3.4	15.7	14.1	96.6	104.3	114.1	5.2	19.5	11.9	110.2	100.5	103.9
Ureidopropionic acid	7.3	15.2	13.9	98.7	104.8	118.3	10.2	19.3	10.2	108.2	98.3	88.4
Uridine	7.6	1.4	10.4	108	101.4	89.6	7.7	1.1	14.5	92.3	91.9	95.5
Uridine diphosphate-N-acetylglucosamine	5.8	BLLOQ	BLLOQ	87.5	BLLOQ	BLLOQ	4.3	BLLOQ	BLLOQ	99.3	BLLOQ	BLLOQ
Uridine diphosphoglucuronic acid	12	13.9	11.2	112	106.1	131.2	10.2	11.1	15.8	99.8	96.9	105.8
Uridine-5′-diphosphogalactose	5.8	BLLOQ	BLLOQ	106	BLLOQ	BLLOQ	7.5	BLLOQ	BLLOQ	99.4	BLLOQ	BLLOQ
Uridine-5'-diphosphate	5.5	7.2	11	106	92.8	111	7.7	6.7	10.3	90.3	103.3	102.3
Uridine-5'-monophosphate	10.4	17.3	14.4	110	102.7	104.4	14.3	19.0	16.9	95.7	105.0	116.9
Xanthine	7.8	18.6	19.5	92.8	102.6	109.5	10.8	14.5	13.6	102.8	94.5	113.6
β-Nicotinamide adenine dinucleotide(NAD)	9.9	BLLOQ	BLLOQ	111	BLLOQ	BLLOQ	8.4	BLLOQ	BLLOQ	88.9	BLLOQ	BLLOQ

BLLOQ: Below limit of quantification.

Table A1.5: Stability of QC samples (25, 250, 750 nM at room temperature (RT) at bench top , 7 days in the refrigerator (4º C) and 30 days storage in the freezer (-20 º C)

Name	Freezer (-20), 30 days (%)			Fridge (4ºC), 7days (%)			Bench top (RT), 6 hrs (%)		
QC level (nM)	750	250	25	750	250	25	750	250	25
17 α-Hydroxypregnenolone	112.8	84.5	114.8	113.3	99.0	108.7	54.9	21.1	122.3
17 α-Hydroxyprogesterone	112.8	74.5	64.8	113.3	89.0	108.7	54.9	21.1	100.2
2,3-Pyridinedicarboxylic acid	111.0	112.8	71.2	128.7	128.1	89.0	20.6	135.9	62.2
21-Deoxycortisol	92.5	BLLOQ	BLLOQ	71.9	BLLOQ	BLLOQ	70.2	BLLOQ	BLLOQ
2-hydroxyglutaric acid	104.5	BLLOQ	BLLOQ	105.2	BLLOQ	BLLOQ	107.9	BLLOQ	BLLOQ
2-Isopropylmalic acid	124.2	84.7	109.8	90.8	103.6	94.3	83.7	88.1	93.7
2-Ketobutyric acid	70.0	62.1	78.9	113.8	67.0	94.5	129.9	65.0	76.2
2-Phosphoglyceric acid	99.4	100.4	99.1	104.7	101.8	97.9	103.5	98.9	99.2
3,4-Dihydroxyphenylacetic acid	103.5	86.8	65.8	116.2	73.7	115.6	168.2	92.7	20.4
3-Hydroxyxanthranilic acid	98.3	BLLOQ	BLLOQ	89.8	BLLOQ	BLLOQ	93.2	BLLOQ	BLLOQ
3-Methylglutaric acid	128.2	90.4	82.3	128.5	113.1	99.4	88.3	76.6	82.4
3-Methylhistidine	106.1	99.9	113.5	114.4	116.7	121.3	120.1	126.3	122.2
3-Phosphoglyceric acid	95.5	BLLOQ	BLLOQ	101.9	BLLOQ	BLLOQ	67.7	BLLOQ	BLLOQ
4,6-Dioxoheptanoic acid	94.3	128.2	96.0	91.1	106.4	68.2	85.1	124.8	98.2
4-Hydroxy-L-glutamic acid	77.3	119.9	79.6	84.3	124.8	66.8	87.3	151.2	115.9
4-Hydroxyphenylpyruvic acid	98.7	BLLOQ	BLLOQ	101.8	BLLOQ	BLLOQ	81.3	BLLOQ	BLLOQ
4-Hydroxyproline	107.4	90.9	95.6	110.4	93.0	84.5	113.0	86.6	78.4
5,6-Dihydro-5-methyluracil	74.0	BLLOQ	BLLOQ	84.0	BLLOQ	BLLOQ	93.5	BLLOQ	BLLOQ
5,6-Dihydrouridine	122.6	85.6	66.5	121.2	68.9	98.4	106.2	102.0	67.6
5-Aminolevulinic acid	68.9	78.6	BLLOQ	78.4	68.4	BLLOQ	28.4	68.9	BLLOQ
5-Hydroxyindoleacetic acid	60.1	BLLOQ	BLLOQ	107.5	BLLOQ	BLLOQ	88.2	BLLOQ	BLLOQ
5-Hydroxylysine	65.8	93.0	102.2	116.7	132.1	92.1	88.2	133.1	109.0
6-Phosphogluconic acid	88.6	102.0	112.3	112.7	101.0	98.3	71.9	52.3	33.4
Acetyl-CoA	90.3	BLLOQ	BLLOQ	108.5	BLLOQ	BLLOQ	52.3	BLLOQ	BLLOQ
Adenine	78.2	85.6	102.0	93.8	88.8	105.0	80.7	94.9	44.5
Adenosin	79.5	198.6	65.0	118.7	121.3	60.5	54.1	181.3	32.8
Adenosine diphosphate	114.4	128.8	83.6	118.1	105.4	80.0	41.3	24.3	18.6
Adenosine monophosphate	120.5	108.8	88.0	110.1	78.7	88.0	149.8	84.7	18.2
Adenosine triphosphate	114.3	120.0	128.0	117.2	117.6	113.0	99.3	82.1	128.0
Adenylosuccinic acid	93.3	85.0	78.0	75.0	106.0	66.5	117.7	0.0	22.5
Adonitol	123.4	108.1	79.4	79.3	77.2	64.0	55.5	83.4	163.9
Alanine	105.3	95.8	181.7	125.0	127.4	117.4	132.1	100.8	131.8
Aldosterone	88.6	102.5	116.2	79.2	101.1	115.4	88.6	95.4	81.4

Aminoadipic acid	111.8	105.7	95.5	93.4	85.2	48.2	93.3	77.9	22.3
Arabinose	117.8	127.1	82.8	106.1	113.3	114.4	80.9	0.2	88.0
Argininosuccinic acid	84.2	78.8	122.8	61.8	124.5	114.5	75.6	138.9	108.2
Betaine	81.0	118.8	69.5	181.4	118.4	72.6	134.9	162.3	40.6
Biopterin	110.9	76.5	125.7	72.9	72.8	127.1	122.8	66.5	104.9
Biotin	119.7	62.8	BLLOQ	96.5	56.2	BLLOQ	231.8	42.3	BLLOQ
Cholic acid	74.7	BLLOQ	BLLOQ	120.8	BLLOQ	BLLOQ	113.8	BLLOQ	BLLOQ
Citrulline	112.3	72.7	134.5	103.6	73.6	25.7	122.2	68.5	16.6
Coenzyme A	97.4	BLLOQ	BLLOQ	109.3	BLLOQ	BLLOQ	100.6	BLLOQ	BLLOQ
Corticosterone	101.2	122.8	103.3	90.8	81.0	107.6	70.5	90.0	126.3
Cortisone	127.2	64.4	74.0	113.6	78.2	91.9	108.1	91.2	50.0
Creatinine	119.9	72.4	112.3	120.5	90.7	107.0	28.0	91.2	250.8
Cyclic AMP	101.0	103.6	128.6	82.0	97.9	142.0	84.8	90.5	96.1
Cyclic GMP	111.2	120.0	63.2	99.0	102.0	118.0	197.6	23.8	19.4
Cysteine-S-sulfate	113.1	87.1	101.0	115.4	99.4	68.3	102.3	73.2	73.3
Cytidine	79.0	118.6	124.0	97.3	86.4	80.6	78.0	92.0	68.8
Cytidine monophosphate	115.8	112.1	125.0	89.0	118.0	82.0	118.0	114.0	95.5
Cytidine monophosphate N-acetylneuraminic acid	102.0	BLLOQ	BLLOQ	102.7	BLLOQ	BLLOQ	108.3	BLLOQ	BLLOQ
Cytidine triphosphate	91.1	104.1	95.0	129.4	130.3	88.9	14.0	26.0	132.1
Cytosine	100.2	110.7	97.1	106.4	112.1	116.1	315.5	18920.9	168.7
Cytosine diphosphate	97.8	97.6	89.7	108.2	86.6	105.0	101.3	100.0	62.6
D-Arabitol	97.3	125.0	120.0	109.7	112.3	68.5	71.0	52.3	48.3
D-Aspartic acid	102.0	104.9	114.6	102.7	117.1	91.8	108.3	132.7	143.0
D-Cytosine monophosphate	107.4	BLLOQ	BLLOQ	115.1	BLLOQ	BLLOQ	126.5	BLLOQ	BLLOQ
Deoxyadenosine	102.6	86.5	81.5	173.8	79.1	119.3	89.9	41.2	146.5
Deoxyadenosine monophosphate	97.6	BLLOQ	BLLOQ	108.0	BLLOQ	BLLOQ	108.0	BLLOQ	BLLOQ
Deoxycytidine	105.8	85.7	111.9	71.7	85.4	114.4	98.9	64.6	96.9
Deoxyguanosine	113.1	BLLOQ	BLLOQ	116.7	BLLOQ	BLLOQ	197.5	BLLOQ	BLLOQ
Deoxyinosine	70.6	110.6	128.0	102.9	84.5	85.3	104.8	25.6	15.2
Deoxyribose-5'-phosphate	98.4	118.2	105.0	61.8	88.8	68.0	89.5	105.7	33.4
Deoxyribose-5'-phosphate	35.0	86.3	84.5	120.6	89.3	62.6	128.6	64.3	28.4
Deoxyuridine	128.4	BLLOQ	BLLOQ	106.8	BLLOQ	BLLOQ	113.6	BLLOQ	BLLOQ
D-Erythrose-4-phosphate	118.6	70.7	65.3	288.4	194.7	98.0	114.2	121.0	98.0
Dexamethasone	129.5	106.8	65.0	68.7	88.6	78.5	78.3	58.4	68.5
Dextrose	124.3	106.2	118.0	101.4	80.4	86.9	167.9	120.5	169.9
D-Fructose	120.9	94.3	106.2	79.7	120.7	67.9	125.5	76.4	71.9
D-Glucose	98.1	BLLOQ	BLLOQ	72.1	BLLOQ	BLLOQ	101.6	BLLOQ	BLLOQ
D-Glucuronic acid	93.1	68.6	109.0	72.6	118.3	77.8	99.9	90.0	102.0
D-Glutamic acid	71.6	111.1	150.7	47.1	93.8	133.7	45.0	84.6	151.8
Dihydrouracil	117.9	BLLOQ	BLLOQ	90.0	BLLOQ	BLLOQ	93.6	BLLOQ	BLLOQ
Dihydroxyacetone phosphate	92.7	73.4	68.0	83.5	66.1	65.0	25.4	79.8	48.3
Dimethylallylpyrophosphate	118.7	65.2	98.2	94.1	61.0	88.6	83.4	97.1	29.3
DL-Homocystine	80.6	94.6	142.7	118.2	119.1	115.1	45.2	114.8	127.2
D-Maltose	92.1	95.2	105.3	105.6	120.7	125.8	124.8	96.5	79.5
D-Mannose	122.1	BLLOQ	BLLOQ	79.4	BLLOQ	BLLOQ	151.9	BLLOQ	BLLOQ
D-Mannose-1-phosphate	97.8	110.2	88.1	87.2	107.3	78.3	84.4	151.7	102.2

Dopamine	109.8	68.5	78.2	121.4	84.3	102.0	158.7	93.5	75.3
D-Ribose-5'-phosphate	98.4	68.2	125.2	61.8	88.8	98.0	89.5	105.7	67.5
D-Threitol	111.6	77.1	91.0	119.6	90.2	120.0	113.2	102.4	71.8
D-Tryptophan	101.4	BLLOQ	BLLOQ	97.8	BLLOQ	BLLOQ	90.0	BLLOQ	BLLOQ
Dulcitol	123.9	93.7	110.6	116.2	98.2	105.8	115.2	19.4	61.7
D-Xylitol	79.7	106.4	120.5	81.1	101.3	80.6	58.3	152.5	70.2
D-Xylose	122.3	BLLOQ	BLLOQ	112.3	BLLOQ	BLLOQ	98.3	BLLOQ	BLLOQ
Estradiol	128.2	120.1	117.2	117.6	113.0	114.2	99.3	82.2	189.8
Estriol	91.5	80.3	96.3	88.7	70.3	98.2	115.4	67.2	73.2
Estrone	206.7	275.6	37.5	91.9	62.1	61.4	172.1	38.2	122.6
Ethanolamine	128.2	74.1	82.0	105.2	116.2	65.0	189.4	151.7	87.0
Fructose-1,6-bisphosphate	114.7	92.5	68.0	121.4	81.1	116.3	143.3	99.3	62.3
Fructose-6-phosphate	115.6	103.9	39.7	108.0	87.8	96.2	101.5	85.5	19.8
Fumaric acid	97.4	125.5	BLLOQ	96.8	82.5	BLLOQ	85.7	57.9	BLLOQ
Galactose-1-phosphate	111.7	78.5	122.4	124.2	102.4	114.4	119.8	116.9	127.9
Gamma-aminobutyric acid (GABA)	115.6	230.2	112.5	127.2	113.3	95.5	125.4	92.0	84.0
Gluconolactone	92.7	102.7	116.9	95.0	113.9	27.8	77.4	90.0	110.2
Glucosamine-6-phosphate	126.7	74.8	103.1	108.4	91.8	68.2	85.9	69.4	73.2
Glucose-6-phosphate	111.7	78.5	122.4	124.2	102.4	114.4	119.8	116.9	107.9
Glutaric acid	91.8	86.7	85.6	83.7	105.6	90.4	47.4	48.4	90.4
Glutathione	114.1	BLLOQ	BLLOQ	111.7	BLLOQ	BLLOQ	0.8	BLLOQ	BLLOQ
Glyceric acid	95.5	120.0	66.0	101.9	94.8	66.5	67.7	65.3	38.2
Glycerol	96.3	112.4	91.1	108.4	106	131	112.6	197.2	112
Glycine	98.0	86.4	95.8	110.1	64.6	92.8	110.7	121.8	127.8
Glycolic acid	100.7	BLLOQ	BLLOQ	107.6	BLLOQ	BLLOQ	115.6	BLLOQ	BLLOQ
Glycoursodeoxycholic acid	98.6	122.0	90.9	124.7	79.1	128.8	109.6	60.5	45.6
Guanine	109.3	106.9	97.4	123.6	108.1	96.8	199.4	205.9	88.6
Guanosine	82.2	BLLOQ	BLLOQ	95.7	BLLOQ	BLLOQ	112.3	BLLOQ	BLLOQ
Guanosine diphosphate	168.3	34.6	58.5	86.4	96.9	90.0	62.4	105.9	42.3
Guanosine monophosphate	12.4	68.2	72.6	68.4	142.3	90.0	112.3	78.5	60.6
Guanosine triphosphate	103.3	97.5	100.4	102.9	107.8	114.7	122.9	103.1	119.9
Homocitrulline	96.9	104.7	113.7	113.6	122.9	117.5	177.4	105.2	150.6
Homo-L-arginine	102.5	93.8	85.6	104.3	111.2	98.6	100.4	100.5	55.4
Homovanillic acid	87.1	108.1	90.0	106.6	105.2	100.0	149.8	116.5	90.0
Hydroxykynurenine	121.0	81.8	97.8	98.0	105.0	109.0	90.0	104.0	103.5
Hypoxanthine	97.8	111.2	70.7	66.0	112.2	97.9	107.5	81.1	120.3
Indoleacetic acid	99.1	120.0	101.9	116.2	116.6	113.4	76.9	106.9	89.4
Inosine	123.9	117.0	91.3	100.0	112.6	125.6	92.1	77.8	105.9
Inosine diphosphate	123.6	112.1	89.2	111.8	85.5	120.0	94.6	76.8	52.5
Inosine triphosphate	82.4	BLLOQ	BLLOQ	129.0	BLLOQ	BLLOQ	94.0	BLLOQ	BLLOQ
Isopentenyl pyrophosphate	104.9	88.3	108.0	143.0	108.0	65.2	125.1	82.3	92.8
Isoxanthopterin	72.3	84.1	122.4	129.0	111.2	125.7	99.3	52.0	90.0
Itaconic acid	98.1	108.9	94.2	105.0	122.1	113.2	82.3	105.1	100.2
L-Acetylcarnitine	82.3	BLLOQ	BLLOQ	122.5	BLLOQ	BLLOQ	157.3	BLLOQ	BLLOQ
L-Alloisoleucine	115.8	BLLOQ	BLLOQ	127.5	BLLOQ	BLLOQ	106.5	BLLOQ	BLLOQ
L-Arginine	102.3	105.9	124.7	111.8	112.0	148.5	121.5	79.7	80.9
L-Asparagine	101.9	89.8	106.7	99.3	95.2	87.9	97.8	85.7	91.3

L-Carnitine	125.7	81.8	115.6	113.4	100.2	118.3	148.9	105.2	93.3
L-Cystathionine	113.8	102.8	100.5	112.4	91.1	109.4	100.7	100.2	26.4
L-Cystine	108.1	104.8	130.1	173.8	116.0	348.2	102.9	95.9	136.2
L-Dihydroorotic acid	98.3	BLLOQ	BLLOQ	82.4	BLLOQ	BLLOQ	75.1	BLLOQ	BLLOQ
L-Galactono-1,4-lactone	95.8	105.3	122.0	116.9	128.0	77.9	106.2	62.3	52.6
L-Glutamine	97.5	102.2	68.7	98.5	128.0	63.7	92.2	114.9	87.7
L-Histidine	102.3	107.7	123.9	113.8	125.4	126.3	125.4	141.2	177.2
L-Homoserine	117.4	29.5	65.3	125.3	73.6	88.0	80.3	128.6	62.3!
L-Isoleucine	112.8	96.3	113.0	106.3	98.9	80.1	95.7	81.4	81.6
Lithium acetoacetate	103.2	92.8	263.3	92.4	88.2	107.6	80.5	30.0	122.6
L-Kynurenine	98.3	84.0	104.2	74.6	62.5	99.8	148.9	482.9	48.2
L-Lactic acid	127.3	BLLOQ	BLLOQ	124.1	BLLOQ	BLLOQ	90.7	BLLOQ	BLLOQ
L-Leucine	118.2	98.9	92.3	116.1	86.8	76.8	141.9	101.3	88.4
L-Lysine	95.4	97.2	110.8	102.3	121.2	109.3	99.9	138.3	138.2
L-Methionine	118.4	108.1	102.3	174.4	122.1	90.6	169.1	119.4	104.1
L-Phenylalanine	101.4	BLLOQ	BLLOQ	105.3	BLLOQ	BLLOQ	96.8	BLLOQ	BLLOQ
L-Proline	123.3	BLLOQ	BLLOQ	97.7	BLLOQ	BLLOQ	106.8	BLLOQ	BLLOQ
L-Ribulose	103.3	BLLOQ	BLLOQ	148.7	BLLOQ	BLLOQ	97.6	BLLOQ	BLLOQ
L-Serine	125.0	98.0	68.7	111.6	81.3	94.5	99.6	73.4	73.9
L-Sorbose	100.3	84.6	119.9	156.4	113.2	60.0	158.7	151.0	121.0
L-threonine	132.8	131.4	71.2	143.9	111.3	99.0	130.0	104.0	46.8
L-Tyrosine	109.4	90.6	97.8	115.0	98.2	75.5	130.6	97.3	80.1
L-Valine	133.8	84.7	100.8	125.7	85.5	80.8	121.6	86.5	93.3
Malic acid	104.5	BLLOQ	BLLOQ	101.3	BLLOQ	BLLOQ	103.7	BLLOQ	BLLOQ
Malonic acid	100.1	122.8	121.4	107.5	124.7	122.3	88.2	148.9	114.2
Mannitol	104.9	BLLOQ	BLLOQ	96.4	BLLOQ	BLLOQ	96.2	BLLOQ	BLLOQ
Melibiose	150.0	197.7	73.7	81.1	112.4	109.5	108.1	95.9	97.7
Methylmalonic acid	92.8	82.9	92.3	118.4	72.8	62.8	134.2	191.2	68.7
Mevalonic acid 3-phosphate	121.6	88.0	127.8	128.5	118.0	104.9	73.1	158.2	95.0
Mevalonic acid-5-phosphate	107.2	99.3	98.2	118.4	98.2	120.0	107.7	74.2	68.6
Monapterin	115.6	124.2	106.6	84.1	107.1	120.0	80.1	97.9	80.8
Myoinositol	92.6	112.0	106.0	92.0	68.6	98.2	65.6	20.3	42.6
N-Acetyl-D-glucosamine	76.1	82.5	98.2	93.2	99.7	78.5	131.0	15.0	12.3
N-Acetyl-D-glucosamine-6-phosphate	112.4	BLLOQ	BLLOQ	70.4	BLLOQ	BLLOQ	123.2	BLLOQ	BLLOQ
N-Acetylmannosamine	83.1	67.2	BLLOQ	103.2	61.7	BLLOQ	16.3	13.4	BLLOQ
N-Acetylneuraminic acid	115.0	77.2	124.2	103.7	78.1	95.7	135.3	76.5	140.9
N-Acetylputrescine	97.4	99.3	109.0	105.8	91.0	111.7	95.3	102.9	98.4
N-Acetylspermine	88.9	92.3	74.5	103.7	94.3	106.7	104.8	56.4	5.4
NADH	97.8	97.6	89.7	108.2	86.6	85.0	101.3	100.0	72.6
Neopterin	70.4	90.1	127.4	91.5	96.9	128.5	78.0	46.4	76.2
Niacinamide	107.7	BLLOQ	BLLOQ	103.1	BLLOQ	BLLOQ	129.1	BLLOQ	BLLOQ
Nicotinamide ribotide	92.7	101.0	102.3	98.7	102.2	101.6	110.1	104.2	78.6
Nicotinic acid	97.0	BLLOQ	BLLOQ	119.3	BLLOQ	BLLOQ	118.9	BLLOQ	BLLOQ
Nicotinic acid mononucleotide	96.5	60.2	114.2	125.6	92.0	98.2	105.2	22.8	16.8
O-Phosphoethanolamine	128.1	BLLOQ	BLLOQ	115.6	BLLOQ	BLLOQ	167.2	BLLOQ	BLLOQ
Ornithine	98.9	96.5	73.7	100.2	91.3	69.0	95.9	64.3	94.3

Orotic acid	85.3	112.1	122.0	117.6	102.0	79.8	67.2	62.3	58.2
Orotidine-5'-monophosphate	82.4	109.2	BLLOQ	61.8	104.8	BLLOQ	74.3	120.3	BLLOQ
O-Succinyl-L-homoserine	88.5	129.0	152.3	88.6	106.2	99.2	44.8	32.8	22.8
Oxalacetic acid	82.3	BLLOQ	BLLOQ	105.6	BLLOQ	BLLOQ	120.3	BLLOQ	BLLOQ
Oxalic acid	81.5	91.1	BLLOQ	117.1	143.4	BLLOQ	139.1	120.3	BLLOQ
Oxidized glutathione	68.2	BLLOQ	BLLOQ	101.2	BLLOQ	BLLOQ	98.6	BLLOQ	BLLOQ
Oxoglutaric acid	115.0	BLLOQ	BLLOQ	98.9	BLLOQ	BLLOQ	102.6	BLLOQ	BLLOQ
Pantothenic acid	105.3	114.7	BLLOQ	118.6	121.9	BLLOQ	10.8	108.5	BLLOQ
Phenylpyruvic acid	88.7	BLLOQ	BLLOQ	101.8	BLLOQ	BLLOQ	81.3	BLLOQ	BLLOQ
Phosphoenolpyruvic acid	115.0	107.4	95.2	113.4	122.1	78.0	110.9	10.5	102.0
Phosphoserine	120.0	112.0	BLLOQ	116.9	65.0	BLLOQ	55.3	23.5	BLLOQ
Pipecolic acid	81.4	97.3	88.2	128.2	94.0	122.0	163.4	115.0	14.2
Porphobilinogen	126.9	BLLOQ	BLLOQ	103.6	BLLOQ	BLLOQ	109.2	BLLOQ	BLLOQ
Progesterone	121.6	198.7	67.5	117.9	129.8	77.4	97.1	94.4	15.2
Pterin	94.5	50.1	162.8	85.4	115.8	128.1	119.0	69.0	90.0
Putrescine	65.4	86.9	76.9	63.8	86.3	83.6	58.8	49.5	196.3
Pyridoxal Hydrochloride	114.7	99.5	101.5	108.4	94.9	101.0	107.4	99.2	148.2
Pyridoxal-5'-phosphate	113.1	84.8	122.0	115.7	105.9	114.1	105.7	108.0	96.0
Raffinose	124.6	115.8	98.4	89.8	103.8	92.8	93.2	128.4	134.8
Ribitol	86.1	85.9	BLLOQ	84.6	82.4	BLLOQ	95.8	105.0	BLLOQ
Saccharopine	83.8	107.2	85.2	57.2	85.9	100.8	53.5	99.5	23.2
Sarcosine	96.0	BLLOQ	BLLOQ	101.1	BLLOQ	BLLOQ	97.0	BLLOQ	BLLOQ
Sedoheptulose-7-phosphate	90.1	129.8	78.3	80.1	67.8	77.8	70.5	102.9	53.2
Sepiapterin	92.9	81.6	68.0	77.4	126.3	68.2	56.2	94.2	68.2
Sodium 4-methyl-2-oxovalerate	111.0	BLLOQ	BLLOQ	126.4	BLLOQ	BLLOQ	121.0	BLLOQ	BLLOQ
Sodium glycodeoxycholate	113.8	116.5	117.0	102.5	103.5	91.8	88.2	95.4	91.6
Sodium β-hydroxyisobutyrate	76.8	BLLOQ	BLLOQ	93.2	BLLOQ	BLLOQ	125.1	BLLOQ	BLLOQ
Sorbitol	117.9	108.3	94.9	103.8	155.7	683.5	119.8	84.3	107.8
Spermine	93.3	79.0	80.5	88.6	91.0	104.2	91.9	16.7	52.2
Sphingosine	188.4	BLLOQ	BLLOQ	129.2	BLLOQ	BLLOQ	280.0	BLLOQ	BLLOQ
S-Sulfocysteine	128.5	119.2	81.4	103.9	124.9	92.5	121.9	113.2	113.1
Succinic acid	74.4	BLLOQ	BLLOQ	85.8	BLLOQ	BLLOQ	72.2	BLLOQ	BLLOQ
Taurine	105.8	85.7	131.9	71.7	85.4	174.4	98.9	64.6	96.9
Taurodeoxycholic acid	115.0	BLLOQ	BLLOQ	103.7	BLLOQ	BLLOQ	135.3	BLLOQ	BLLOQ
Tauroursodeoxycholic Acid,	28.3	66.4	28.3	112.3	96.5	88.3	113.6	101.9	90.5
Thiamine	78.6	99.9	65.2	112.0	65.3	88.0	90.0	109.8	42.3
Thymidine	106.5	122.5	64.2	114.7	120.0	118.0	121.1	123.9	77.0
Thymine	115.8	130.6	98.0	94.1	68.3	77.8	121.8	101.3	25.3
Tryptophanol	128.0	BLLOQ	BLLOQ	122.8	BLLOQ	BLLOQ	46.9	BLLOQ	BLLOQ
Uracil	111.2	105.3	95.3	126.6	125.2	63.5	70.1	91.4	96.6
Ureidopropionic acid	120.0	122.0	67.0	105.8	106.5	99.8	112.8	111.6	108
Uridine	110.3	120.1	122.0	89.7	125.8	61.1	84.0	65.0	91.9
Uridine diphosphate-N-acetylglucosamine	85.7	BLLOQ	BLLOQ	109.3	BLLOQ	BLLOQ	68.6	BLLOQ	BLLOQ
Uridine diphosphoglucuronic acid	105.8	122.2	95.4	95.9	101.2	68.9	68.2	88.2	118.0

Uridine-5′-diphosphogalactose	124.3	BLLOQ	BLLOQ	121.4	BLLOQ	BLLOQ	167.9	BLLOQ	BLLOQ
Uridine-5'-diphosphate	83.3	96.6	90.6	89.9	111.4	75.9	28.3	15.3	4.8
Uridine-5'-monophosphate	92.8	127.0	125.0	92.2	79.4	82.6	73.9	96.0	109.1
Xanthine	108.3	120.2	108.0	127.6	116.0	65.8	124.3	73.1	68.0
β-Nicotinamide adenine dinucleotide(NAD)	128.7	BLLOQ	BLLOQ	79.7	BLLOQ	BLLOQ	125.6	BLLOQ	BLLOQ

Table A1.6: Long-term stability of detected metabolites in DBS after 2 and 4 months at 4℃

Metabolite	2 Months (%)	4 Months (%)
17 α-Hydroxypregnenolone	77	88.5
17 α-Hydroxyprogesterone	111.38	92.62
2-Dexoycytidine	97.42	84.11
2-Hydroxyglutaric acid	75.27	77.5
2-ketobutyric acid	122.71	103.84
2-Oxo-glutaric acid	76.52	75.59
2-Phoshoglyceric acid	130.56	135.89
3,4-Dihydrophenylacetic acid	88.73	108.81
3-Indoleacetic acid	77.9	59.91
3-Methylglutaric acid	88	79.23
3-Methylhistidine	100.37	67.11
3-phoshoglyceric acid	66.03	77.55
3-Ureidopropionic acid	69.28	68.59
4,6-Dioxoheptanoic acid (Succinylacetone)	63.12	61.75
4-Hydroxy-L-glutamic acid	103.33	63.81
4-Hydroxyproline	38.05	103.84
5-Aminoleuvillinic acid	64.38	81.68
5-Hydroxyindole-3-acetic acid	75.64	70.14
5-Hydroxylysine HCL	105.16	51.62
Adenosine	124.19	101.04
Adenosine-3',5'-monophosphate (cAMP)	89.36	99.78
Alanine	46.14	65.35
Aminoadipic acid	57.24	52.38
Arabinose	98	55.36
Arabitol	101.79	54.22
Arginine	82.88	66.34
Asparagine	40.46	60.97
Aspartic acid	70.87	59.75
Betaine	58.28	90.44
Biotin	104.57	95.4
Citrulline	102.88	130.5
Corticosterone	116.88	98.49
Cortisone	83.82	91.83
Creatinine	45.37	100.5
Cysteine sulphate	88.82	66.29
Cystine	90.26	101.13
Cytidine-5-triphoshate (CTP)	85.2	69.63
Cytidne-5-diphosphate (CDP)	103.95	73.54
Cytosine	85.47	72.21

Dextrose	108.12	57.78
D-Galactose-1-phosphate	133.75	102.45
Dihydroxyacetone phosphate	109.49	77.56
Diydhyrouracil	57.6	62.37
Dopamine	110.82	92.21
D-Panthothenic acid	106.4	85.46
D-Ribose-5-Phosphate	84.94	60.97
Estradiol	107.05	89.5
Ethanolamine	114.29	93.58
Fructose	102.12	92.57
Fructose-6-phosphate	70.78	139.07
Gamma aminobutyric acid (GABA)	75.12	105.84
Glucosamine-6-phosphate	63.91	87.55
Glucose-6-phosphate	67.39	51.62
Glucuronic acid	78.89	100.37
Glutamic acid	90.37	103.84
Glutamine	92.79	105.2
Gluthathione	103.83	81.42
Glyceric acid	105.77	93.68
Glycine	67.81	99.29
Glycolic acid	91.43	106.4
Glycursodeoxycholic acid	67.81	78.87
Guanine	76.83	81.29
Guanosine	81.91	87.89
Histidine	99.29	77.44
Homoarginine	83.14	90.7
Homocitrulline	81.49	11.14*
Homocystine	99.29	106.59
Homovanillic acid	69.52	102.26
Hypoxanthine	104.68	113.25
Inosine	88.01	64.28
Inosine 5'-diphosphate (IDP)	50.72	57.28
Isoleucine	65.23	90.37
Isopropyl malic acid	56.8	115.54
Itaconic (2-methylenesuccinic) acid	89.16	81.56
Kynurenine	78.54	95.02
L-Carnitine	120.91	120.46
Leucine	124.19	92.79
Lysine	90.37	75.91
Malic acid	71.78	93.33
Malonic acid	71.95	55.13
Mannose	103.5	72.53
Mannose-1-phosphate	103.58	106.57
Methionine	105.84	66.34

Methylmalonate (MMA)	87.37	100.58
Mevalonate-5-phosphate	65.08	90.15
N-Acetyl-D-mannosamine	109.39	80.87
N-Acetylglucosamine	99.84	70.44
N-Acetyl-glucosamine-1-phosphate	65.08	105.16
N-Acetylneuraminic acid (Sialic acid)	56.14	77.44
N-Acetylspermine	80.77	81.65
Niacinamide	97.5	114.16
Nicotinamide ribotide	106.43	103.4
Nicotinic acid	57.44	130.5
Nicotinic acid mononucleotide	110.06	94.03
O-Acetyl-carnitine	74.6	88.35
O-Phoshoserine	117.02	112.64
O-Phosphoserine	111.97	118.31
Ornithine	89.36	66.29
Orotic acid	103.32	91.41
Oxalic acid	98.03	82.3
Oxaloacetate	68.77	149.31
Phenylalanine	33.45	102.45
Phosphoenolpyruvic acid	71.82	87.77
Phosphoethanolamine	64.16	82.56
Pipecolic acid	89.22	90.69
Progesterone	111.26	102.64
Proline	81.91	100.37
Raffinose	95.41	79.66
Ribitol (adonitol)	75.33	76.42
Ribulose	98.71	62.81
Saccharopine	110.44	82.47
Sarcosine	5.96	101.51
Sepiaterin	65.6	74.53
Serine	127.66	101.51
Sodium 4-methyl-3-oxovalerate	88.1	77.73
Sodium beta-hydroxy-isobutyrate	32.63	85.35
Sodium glycodeoxycholate	81.67	21.06
Sodium taurodeoxycholate	115.62	10.64*
Sorbose	78.54	149.22
Spermine	53.05	67.11
S-Sulfocysteine	98.03	84.76
Succinic acid	96.42	72.26
Taurine	130.44	67.11
Threitol	98.55	69.37
Threonine	88.01	182.88
Tyrosine	92.79	101.51
Tyrptophan	105.16	92

Uridine	65.23	102.83
Uridine 5-diphospho-N-acetylglycosamine	93.79	66.34
Valine	105.84	115.23
Xanthine	75.12	57.81
Xylose	104.12	83.57

Table A1.7: Extraction recovery evaluation from serum samples based on selected labeled internal standards

Internal standards	**Recovery (%)**
2-Deoxyadenosine-C13	61.99
Alanine-d4	65.45
Arginine-d7	54.29
Aspartate-d3	71.93
C 16 Carnitine-d3	89.5
C10 Carnitine-d3	79.26
C12 Carnitine-d3	68.51
C14 Carnitine-d3	102.94
C18 Carnitine-d3	125.43
C4 Carnitine-d3	122.94
C5 Carnitine-d9	86.6
C6 Carnitine-d3	87.12
C8 Carnitine-d3	71.73
C0 Carnitine-d9	98.95
Citrulline-d2	84.35
Fructose(2-13C)	127.76
Glucose-d7	89.96
Glutamate-d5	88.95
Leucine-d3	73.72
Methionine-d3	55.62
Ornithine-d6	82.48
Phenylalanine-d5	97.83
Tyrosine-d4	79.73
Valine-d8	56.78

Table A1.8: List of DBS metabolites with the reference ranges, the reference interval was calculated using non-parametrical percentile method

Metabolite	Average (nM)	95%
2-Deoxyadenosine	20.60	5.17
2-Deoxycytidine 5 monophosphate	18.26	3.6
2-Deoxyinosine	55.52	7.65
2-Deoxyribose 5 phosphate	135.53	24.07
2-ketobutyric acid	160.92	7.06
3,4, Dihydrophenylacetic acid	207.89	67.44
3-ureidopropionic acid	116.93	9.25
4,6,Dioxoheptanoic acid	6.10	1.54
4-Hydroxyglutamic aicd	133.31	24.51
4-Hydroxy-proline	169.66	35.48
Adenine	74.21	9.86
Adenosine	154.96	5.70
Adenosine 3,5 monophosphate(cAMP)	18.10	3.37
Adenosine 5 diphosphate(ADP)	86.28	7.92
Adenosine 5 triphosphate(ATP)	20.925	6.87
Adenosine monophosphate(AMP)	43.54	7.77
Adenylosuccinic acid	34.11	6.48
Alanine	39.88	7.66
Aminoadipic	14.13	4.07
Arabitol	58.58	4.77
Arginine	124.68	6.28
Asparagine	301.93	6.39
Aspartic acid	276.07	5.23
Betaine	201.49	9.37
Biopterin	19.07	4.48
cGMP	51.91	13.12
Citrulline	52.45	10.70
Corticosterone	13.61	3.81
Cortisone	48.44	2.25
Creatinine	92.90	17.61247
Cysteine sulphate	12.25	0.413485
Cysthathionine	19.11	5.91
Cystine	366.14	94.16
Cytidine	40.78	6.71
Cytidine 5 triphoshate(CTP)	13.85	2.25
Cytidine monophosphate(CMP)	93.44	38.40
Cytidne 5 diphosphate(CDP)	51.92	10.39

Cytosine	134.75	37.04
D-Galactose 1 phosphate	179.15	9.04
Dihydroxyacetone Phosphate	327.57	24.86
dopamine	84.69	11.31
D-Ribose 5 Phosphate	347.04	23.54
D-Sedoheptulose 7 phosphate	139.01	21.03
Erythrose 4 phosphate	371.78	48.76
Estradiol	113.96	3.73
Estrone	56.88	2.28
Ethanolamine	108.25	18.50
Fructose 1 6 Bisphosphate	456.08	34.77
Fructose 6 phosphate	377.25	67.23
Gluconic acid lactone	27.74	3.62
Glucose 6 Phosphate	204.61	85.07
Glucuronic acid	193.07	4.92
Glutamic acid	2279.93	85.81
Glutamine	976.22	103.95
Glycerol	974.46	45.39
Glycine	171.28	7.09
Glycolic acid	168.43	40.19
GMP	119.64	2.09
Guanine	327.79	74.76
Guanosine	298.11	76.85
Guanosine di phosphate(GDP)	119.73	37.37
Guanosine triphosphate(GTP)	28.76	88.80
Histidine	94.26	5.03
Homoarginine	269.58	38.81
Homocystine	43.80	10.65
Hypoxanthine	429.55	95.55
Inosine	43.56	8.94
Inosine 5 diphosphate(IDP)	71.87	21.84
Isoleucine	187.48	6.57
Isopentyl pyrophoshate	273.62	9.16
Isoprophymallic	3.79	0.56
Isoxanthopterin	28.75	4.00
Kynurenine	58.63	8.57
L-Carnitine	274.95	48.53
Leucine	171.14	5.35
Lysine	433.13	85.58
Malonic acid	38.50	9.85
Mannose 1 phosphate	136.13	14.00
Methionine	197.26	5.39
Monapterin	12.95	4.06
N-Acetylneuraminic acid	184.05	28.37

N-Acetylspermine	18.08	5.23
NADH	75.11	12.96
NADP	30.87	2.82
Neopterin	44.24	7.00
Nicotinic acid	39.69	7.75
O-Phoshoserine	185.06	10.57
Ornithine	11.51	3.09
Orotic acid	179.35	4.71
Phenylalanine	2153.58	42.174
Phosphoenolpyruvic acid	292.76	18.16
Pipecolinic acid	61.50	81.73
Progesterone	848.24	160.24
pterin	23.45	3.40
putrescine	130.38	27.42
Saccharopine	23.05	6.08
Serine	387.16	31.86
Spermine	88.64	53.10
Sulfocystiene	15.21	1.715252
Taurine	144.98	40.35
Thiamine	240.91	26.88
Threitol	57.17	9.45
Threonine	850.63	17.87
Thymidine	22.81	5.56
Thymine	5.61	1.20
Tyrosine	829.63	53.74
Tyrptophan	419.64	10.18
Uracil	15.58	3.36
Uridine	82.75	25.74
Uridine 5 monophosphate(UMP)	175.97	9.5
Uridine di phosphate(UDP)	11.51	3.08
Valine	422.27	10.61
Xanthine	196.32	42.14
Xylitol	37.85	7.73
Y Y Dimethylallyl pyrophoshate	168.84	5.78

Table A1.9: Summary of clinical scores (VAS and SCORAD) and laboratory findings in DOCK8 deficient and atopic dermatitis cohorts

Diagnosis	Patient code	Mutation	Age (Y)	Sex (M/F)	IgE Levels (KU/L)	RBC 10^ 12/L	WBC 10^9/L	Eosinophils 10^9/L	CD4/ CD8 Ratio	VAS	SCORAD
DOCK8	P1	NM_203447.3:c.[2606-1G>A; c.405_827del]	21	F	25000	0.01	9.2	24.8	2.1	15	68.7
	P2	NM_203447.3:c.[2606-1G>A; c.405_827del]	16	M	9140	4.17	3.72	35	0.8	13	67
	P3	NM_203447.3:c.5625T>G:p.Y1875	16	F	1690	4.2	4.63	50.3	1	14	59.8
	P4	NM_203447:c.827+6T>C	16	F	11240	5.9	4.9	11.4	0.73	15	77.4
	P5	NM_203447.3:c.5962_6068del	7	M	10170	5.29	9.93	26.4	0.7	17	79.5
	P6	NM_001193536:c.3949+1G>T	14	M	15550	6.82	4.46	11.7	1.3	15	62.5
	P7	NM_001193536:c.1593+1G>T	4	M	86940	5.3	24.9	31.4	9	17	70.6
	P8	NM_001193536:c.1593+1G>T	9	F	44630	5.26	12.49	34.8	8.1	9	58.2
	P9	NM_001190458:c.1905_1905+1delGG	8	F	26340	3.81	21.42	35.1	3.2	14	58.8
	P10	NM_203447.3:c.5625T>G:p.Y1875	21	M	265	4.7	9.7	12	1.1	12	70.1
Average±SEM			**13.2±5.9**	**5/5 (M/F)**	**19817.30±4772.6**	**4.5±0.57**	**10.53±2.3**	**27.29±4.02**	**2.8±0.99**	**14.1±0.75**	**67.26 ±2.37**
ATOPIC DERMATITIS	P11	**DOCK8 mutation negative**	6	M	6540	5.2	10.6	16.5	0.9	16	64.4
	P12		15	M	16500	6.39	3.72	15.6	1.4	14	71.3
	P13		10	M	1612	5.48	8.01	13.9	1.2	16	73.3
	P14		3	M	839	5.3	6.59	6.7	_	13	56.1
	P15		8	M	1221	4.9	5.09	13	1.5	9	40.8
	P16		13	M	659	5.2	5.15	13	1.2	10	54.9
	P17		15	F	1387	4.71	9.63	12.3	1.4	15	75
	P18		16	M	365	5.15	2.71	7.9	2.3	14	65.1
	P19		12	M	4587	5.66	9.2	5.1	1.5	16	70.6
Average±SEM			**10.8±1.4**	**8/1 (M/F)**	**5288.20±1736.3**	**5.33±0.16**	**6.74±0.92**	**27.29±1.3**	**1.43±0.14**	**13.6±0.86**	**63.5 ± 3.702**

Table A1.10: Positively identified (DOCK8 Vs Control)

HMDB.No	Name	Acc.mass	mz_light	Retention time	Fold.change	p value
316	Phenyl-Leucine	278.1631	512.2214	15.9	0.395277836	0.0000603
HMDB00020	p-Hydroxyphenylacetic acid	152.0473	386.1057	16.91	1.665531722	0.0008974
HMDB00070	D-Pipecolic acid	129.079	363.1373	13.23	1.587058087	2.12E-04
HMDB00112	Gamma-Aminobutyric acid	103.0633	337.1216	7.79	1.534256238	4.43E-10
HMDB00133	Guanosine	283.0917	517.15	2.22	4.122263032	8.87E-07
HMDB00206	N6-Acetyl-L-Lysine	188.1161	422.1744	5.71	1.681202991	6.36E-10
HMDB00210	Pantothenic acid	219.1107	453.169	8.37	1.832101063	1.06E-11
HMDB00224	O-Phosphoethanolamine	141.0191	375.0774	2.02	0.582279964	7.63E-05
HMDB00251	Taurine	125.0147	359.073	2.24	0.628274512	7.67E-08
HMDB00259	Serotonin	176.095	322.1058	24.65	1.815645678	3.08E-06
HMDB00271	Sarcosine	89.0477	323.106	9.34	1.573575643	8.23E-09
HMDB00300	Uracil	112.0273	346.0856	11.34	2.245592611	3.35E-06
HMDB00440	3-Hydroxyphenylacetic acid	152.0473	386.1057	16.72	1.665531722	8.97E-04
HMDB00450	5-Hydroxylysine	162.1004	315.1085	13.88	2.087090747	2.92E-11
HMDB00452	L-Alpha-aminobutyric acid	103.0633	337.1216	9.13	2.122152479	2.64E-08
HMDB00469	5-Hydroxymethyluracil	142.0378	376.0962	8.87	0.661087618	6.95E-07
HMDB00500	4-Hydroxybenzoic acid	138.0317	372.09	17.57	0.481133034	0.032552
HMDB00500	4-Hydroxybenzoic acid	138.0317	372.09	17.57	0.497054292	1.36E-12
HMDB00669	Ortho-Hydroxyphenylacetic acid	152.0473	386.1057	16.42	1.665531722	8.97E-04
HMDB00684	L-Kynurenine	208.0848	442.1431	11.44	1.744385361	1.07E-10
HMDB00706	L-Aspartyl-L-phenylalanine	280.1059	514.1642	10.07	0.315025661	1.07E-05
HMDB00716	L-Pipecolic acid	129.079	363.1373	13.45	1.587058087	2.12E-04
HMDB00750_2	3-Hydroxymandelic acid - COOH	168.0423	356.0951	21.64	1.586042823	1.04E-13
HMDB00965	Hypotaurine	109.0197	343.0781	2.47	0.437626734	2.36E-05
HMDB01123	2-Aminobenzoic acid	137.0477	371.106	16.62	3.014850957	5.37E-10
HMDB01232	4-Nitrophenol	139.0269	373.0853	23.45	1.730548304	1.06E-11
HMDB01414	1,4-diaminobutane	88.1	278.1083	21.27	2.039329127	1.10E-10
HMDB01476	3-Hydroxyanthranilic acid	153.0426	387.1009	18.14	1.808714598	8.01E-10
HMDB01906	2-Aminoisobutyric acid	103.0633	337.1216	8.91	2.122152479	2.64E-08
HMDB01918	Thyroxine	776.6867	622.4017	27.74	1.639578496	2.11E-13
HMDB02064	N-Acetylputrescine	130.1106	364.1689	7.25	2.196515071	3.24E-14
HMDB02390	3-Cresotinic acid	152.0473	386.1057	16.8	1.665531722	8.97E-04
HMDB03012	Aniline	93.0578	327.1162	17.32	2.035166282	3.6E-11
HMDB03334	Symmetric dimethylarginine	202.143	436.2013	3.05	1.92465467	1.95E-17
HMDB03355	5-Aminopentanoic acid	117.079	351.1373	8.68	1.622041349	7.51E-04
HMDB03911	3-Aminoisobutanoic acid	103.0633	337.1216	8.67	2.122152479	2.64E-08
HMDB03911_2	3-Aminoisobutanoic acid - H2O	103.0633	319.111	16.29	1.943610889	4.66E-07
HMDB13302	Phenylalanylphenylalanine	312.1474	546.2057	16.55	0.529397252	2.43E-07
HMDB28848	Glycyl-Phenylalanine	222.1004	456.1588	11.65	0.597854182	0.000037
HMDB28854	Glycyl-Valine	174.1004	408.1588	9.19	0.266569466	0.0013041

Table A1.11: Positively identified (AD Vs Control)

HMDB.No.	Name	Acc.mass	mz_light	Retention time	Fold.change	p value
316	Phenyl-Leucine	278.1631	512.2214	15.9	0.221545833	8.30E-06
HMDB00130	Homogentisic acid	168.0423	318.0794	24.84	0.512373096	3.44E-06
HMDB00133	Guanosine	283.0917	517.15	2.22	6.402693356	1.20E-12
HMDB00148	L-Glutamic Acid	147.0532	381.1115	5.05	0.534262485	3.31E-07
HMDB00152	Gentisic acid	154.0266	388.0849	17.11	0.546812559	0.0150655
HMDB00191	L-Aspartic Acid	133.0375	367.0958	5.16	0.639508421	0.0004979
HMDB00210	Pantothenic acid	219.1107	453.169	8.37	1.539230701	4.48E-07
HMDB00214	Ornithine	132.0899	300.1033	16.58	0.579056214	3.81E-10
HMDB00224	O-Phosphoethanolamine	141.0191	375.0774	2.02	0.570539227	9.49E-05
HMDB00251	Taurine	125.0147	359.073	2.24	0.620118218	2.20E-07
HMDB00259	Serotonin	176.095	322.1058	24.65	2.828641474	1.96E-12
HMDB00440	3-Hydroxyphenylacetic acid	152.0473	386.1057	16.72	0.660875641	0.0219098
HMDB00450	5-Hydroxylysine	162.1004	315.1085	13.88	2.452586635	2.13E-13
HMDB00452	L-Alpha-aminobutyric acid	103.0633	337.1216	9.13	1.775708991	6.46E-07
HMDB00500	4-Hydroxybenzoic acid	138.0317	372.09	17.57	0.53377388	0.035612
HMDB00517	L-Arginine	174.1117	408.17	2.44	1.616969697	1.74E-14
HMDB00669	Ortho-Hydroxyphenylacetic acid	152.0473	386.1057	16.42	0.660875641	0.0219098
HMDB00706	L-Aspartyl-L-phenylalanine	280.1059	514.1642	10.07	0.189420758	2.00E-06
HMDB00725	Trans-4-Hydroxyl-L-Proline	131.0582	365.1166	5.17	1.556154941	2.61E-05
HMDB00750_2	3-Hydroxymandelic acid - COOH	168.0423	356.0951	21.64	1.571944665	3.85E-13
HMDB00991	2-aminooctanoic acid	159.1259	393.1842	19.2	2.092570984	2.32E-10
HMDB01123	2-Aminobenzoic acid	137.0477	371.106	16.62	1.989599933	6.64E-12
HMDB01232	4-Nitrophenol	139.0269	373.0853	23.45	1.665664174	9.49E-10
HMDB01336	3,4-Dihydroxybenzene acetic acid	168.0423	318.0794	23.9	1.6678413	1.63E-06
HMDB01414	1,4-diaminobutane	88.1	278.1083	21.27	1.80273571	4.66E-07
HMDB01856	Protocatechuic acid	154.02661	311.0716	24.51	0.560950951	0.0521732
HMDB01906	2-Aminoisobutyric acid	103.0633	337.1216	8.91	1.775708991	6.46E-07
HMDB01918	Thyroxine	776.6867	622.4017	27.74	1.624099542	2.76E-10
HMDB02064	N-Acetylputrescine	130.1106	364.1689	7.25	1.798382188	3.06E-14
HMDB02390	3-Cresotinic acid	152.0473	386.1057	16.8	0.660875641	0.0219098
HMDB03012	Aniline	93.0578	327.1162	17.32	3.1256744	0.0009532
HMDB03334	Symmetricdimethylarginine	202.143	436.2013	3.05	1.82325156	1.23E-22
HMDB03355	5-Aminopentanoic acid	117.079	351.1373	8.68	1.623790518	5.53E-10

HMDB03911	3-Aminoisobutanoic acid	103.0633	337.1216	8.67	1.775708991	6.46E-07
HMDB13243	Leucyl-phenylalanine	278.163	512.2214	16.59	1.759357659	1.72E-06
HMDB13302	Phenylalanylphenylalanine	312.1474	546.2057	16.55	0.362381413	9.47E-11
HMDB28854	Glycyl-Valine	174.1004	408.1588	9.19	0.096747134	0.0003284

Table A1.12: Positively identified (DOCK8 vs AD)

HMDB.No.	Name	Accurate mass	mz_light	Retention time	Fold. change	p value
HMDB00133	Guanosine	283.0917	517.15	2.22	1.553198645	0.006756897
HMDB00191	L-Aspartic Acid	133.0375	367.0958	5.16	0.552161486	0.005203074
HMDB00965	Hypotaurine	109.0197	343.0781	2.47	1.898280637	0.000832791
HMDB00991	2-aminooctanoic acid	159.1259	393.1842	19.2	1.550838574	0.005658227
HMDB01476	3-Hydroxyanthranilic acid	153.0426	387.1009	18.14	0.624233115	0.00095519
HMDB13243	Leucyl-phenylalanine	278.163	512.2214	16.59	2.415598554	4.67E-06
HMDB28848	Glycyl-Phenylalanine	222.1004	456.1588	11.65	2.091982277	0.000524798

Table A1.13: ImmunoCAP® ISAC panel (112 allergens)

Aeroallergens (64 sources)	Allergen components				
Horse	rEqu C1	rEqu C3			
Cat	rFel d1	rFel d2	rFel d4		
Mouse	nMus m1				
Alternaria	rAlt a1	rAlt a6			
Aspergillus	rAsp f1	rAsp f3	rAsp f6		
clasdosporium	rCla h8				
House dust mite	rBlo t5	nDer f1	nDer f2	nDer p1	nDer p2
	nDer p10				
storage mite	rLep d2				
Cockroach	rBla g1	rBla g2	rBla g5	rBla g7	
Latex	rHev b1	rHev b3	rHev b5	rHev b6.01	rHev b8
Sugar epitope from Bromelain	Nmuxf3				
Bermuda grass	nCyn d1				
Timothy grass	rPhl p1	rPhl p2	rPhl p4	rPhl p5	rPhl p6
	rPhl p7	rPhl p11	rPhl p12		
Alder	rAln g1				
Birch	rBet v1	rBet v2	rBet v4		
Hazel pollen	rCor a 1.0101				
Japanese ceder	nCry j1				
Cypress	nCup a1				
Olive	nOle e1	nOle e7	nOle e9		
Plane tree	rPla a1	rPla a2	rPla a3		
Ragweed	nAmb a1				
Mugwort	nArtv v1	nArtv v3			
Goosefoot	rChe a1				
Annual mercury	rMer a1				
Wall pellitory	rPar j2				
Plantain(English)	rPla l1				
Salwort	nSal k1				
Dog	rCan f1	rCan f2	rCan f3	rCan f5	

Food Allergens (44 sources)	Allergen components				
Egg	nGal d1	nGal d2	nGal d3	nGal d5	
Milk	nBos d4	nBos d5	nBos d6	nBos d8	nBos dlactoferrin
Shrimp& Cod	nPen m1	nPen m2	nPen m4	Rgad c1	
Peanut	rAra h1	rAra h2	rAra h3	rAra h6	rAra h8
	rAra h9				
Soybean	rGly m4	rGly m5	rGly m6		
Wheat	rTria a 14	rTria a 19.01	rTria a Aa_TI		
Fruits(Apple,peach,Kiwi,celery)	rMal d1	rPru p1	rPru p3	nAct d 1	nAct d 2
	nAct d 5	nAct d 8	rApi g1		
Nuts(walnut,Hazelnut,	nJug r1	nJug r2	nJug r3	rCor a1.040	rCor a8
Brazilnut,cashew nut)	rCor a9	rBer e1	rAna o2		

Others (4 sources)	Allergen components				
Honey bee venom	rApi m1	rApi m4			
Paper wasp venom	rPol d5				
common wasp venom	rVes v5				
Anisakis	rAni s1	rAni s3			

Table A1.14: Analysis of Food-allergens in DOCK8-deficient and AD patients

Food allergens	Common name	Protein name	DOCK8 (n=10)				AD (n=9)			
			%	Median	IQR	Species %	%	Median	IQR	Species %
nGal d1	Egg white	Ovomucoid	50	2.3	1.0	60	44	9.7	6.8	67
nGal d2		Ovalbumin	50	17	13.0		56	1.9	1.1	
nGal d3		Conalbumin	40	7.25	2.9		56	2.4	0.8	
nGal d5		serum albumin	20	9.35	5.0		33	1.2	1.0	
nBos d4	cow's milk	alpha lactalbumin	50	14	8.5	80	22	1.1	1.1	44
nBos d5		beta-lactoglobulin	70	4.8	1.5		22	1.2	0.8	
nBos d6		serum albumin	0	0	0.0		11	9.2	9.2	
nBos d8		caseins	80	6.05	2.4		33	0.5	0.5	
nBos d lactoferrin		lactoferrin	0	0	0.0		11	2.6	2.6	
rGad c1	Cod	parvalbumin	0	0	0.0	0	11	1.4	1.4	11
nPen m2	shrimp	Tropomyosin	0	0	0.0	0	22	24.1	14.1	22
nPen m4		Tropomyosin	0	0	0.0		22	21.2	10.8	
rAna o2	Treenut		70	5.1	2.7	70	56	1.5	0.4	56
rBer e1		storage protien ,2S albumin		0	0.0		44	1.9	0.7	
Cor a9		storage protien ,11S globulin	50	21	2.5		67	0.5	0.4	
nJug r1		2S Albumin, a storage protein	30	3.4	1.9		56	22.0	8.8	
nJug r2		vicilin	40	5.4	2.4		67	1.3	1.1	
nSes i1	sesame	storage protien ,2S albumin	60	26	12.3	60	67	22.0	16.5	67
rAra h1	peanut	storage protien ,vicilin	50	8.4	4.8	60	56	26.0	0.9	56
rAra h2		storage protien ,conglutin	40	20	5.8		44	38.0	19.2	
rAra h3		storage protien,11S globulin	60	3.3	1.0		22	12.6	7.3	
rAra h6		PR-10 protein	30	18	11.1		44	39.0	24.8	
rGly m5	soyabean	storage protien ,beta-conglycinin	50	9.2	2.0	60	33	2.1	1.7	56
rGly m6		storage protien ,glycinin	70	11.9	1.8		56	2.8	0.9	
rTria a 14	wheat		20	16.35	12.5	40	22	1.2	0.8	22
rTria a AaTI			40	9.45	5.3		11	2.8	2.8	

Table A1.15: Analysis of Aero-allergens in DOCK8-deficient and AD patients

Aero allergens	Common name	Protein group	DOCK8				AD			
			%	Median	IQR	Species %	%	Median	IQR	Species %
nCyn d1	barmuda grass	Group 1 Grasses	40.0	2.3	2.0	40	56	5.0	1.7	56
rPhl p1	Timothy grass	Group 1 Grasses	0	0.0	0.0		11	2.1	2.1	56
rPhl p4			50	5.0	1.6	50	56	1.7	1.3	
rPhl p5		Group 5Grasses	10	0.9	0.9		0	0.0	0	
rBet v1	Birch	PR-10 protein	10	6.0	6.0	10	11	0.4	0.4	11
nCry j1	ceder		30	6.6	4.6	30	33	0.8	0.8	33
nCup a1	Cypress		30	2.7	2.5	30	33	0.5	0.5	33
nOle e9	olive		40	3.0	1.9	40	22	0.7	0.7	44
rPla a1	plane tree		10	21.0	21.0	50	0	0.0	0	44
rPla a2			50	2.1	0.7		44	1.2	1.2	
rChe a1	goosefoot	Ole-e-1-like family of proteins	0	0.0	0.0	0	11	4.1	4.1	11
rPar j2	wall pellitory	PR-14) protein family.	30	0.8	0.8	30	0	0.0	0	11
rPla l1	plantain		10	1.3	1.3	10	0	0.0	0	0
nSal k1	Salwort	salsola	0	0.0	0.0	0	11	4.1	4.1	11
rCan f1	Dog	lipocalin	0	0.0	0.0	0	11	3.8	3.8	11
rFel d1	cat	Ulteroglobin	0	0.0	0.0	0	78	43.0	36.8	78
nMus m1	Mouse	lipocalin	10	2.0	2.0	10	0	0.0	0	0
rAlt a1	Alternaria		10	0.4	0.4	40	11	0.6	0.6	22
rAlt a6			30	1.4	1.1		22	4.5	3.4	
rAsp f1	Aspergillus		10	0.6	0.6	10	0	0.0	0	44
rAsp f6			0	0.0	0.0		44	1.2	0.4	
Bla g 5	cockroach		0	0.0	0.0	0	33	14.0	10.7	33
rPol d5	paper wasp venom		10	0.9	0.9	10	11	0.3	0.3	11
rHev b1	Latex		30	0.6	0.6	60	0	0.0	0	0
rHev b3			30	1.1	0.8		0	0.0	0	
rHev b6.01			60	0.8	0.7		0	0.0	0	

Table A1.16 : Disease specific metabolites in DOCK8-deficient patients in different food groups

Disease specific metabolites	Egg	Milk	Peanut	Sesame	Soy
Taurine	D	D	NS	NS	NS
L-threo-3-Methylaspartate	U	NS	NS	NS	NS
L-Aspartate 4-semialdehyde	U	NS	NS	NS	NS
5-Amino-2-oxopentanoic acid	U	NS	NS	NS	NS
Tyrosyl-Asparagine	D	NS	NS	NS	NS
L-2-Amino-3-oxobutanoic acid	U	NS	NS	D	NS
2'-Deamino-2'-hydroxy-6'-dehydroparomamine	U	NS	NS	NS	NS
3-Methoxy-4-hydroxyphenylethyleneglycol	D	NS	NS	NS	NS
2-Aminobenzoic acid	D	NS	NS	D	NS
5-Hydroxyindoleacetic acid	D	NS	NS	NS	NS
2R,5S-2,5-Diaminohexanoate	U	NS	NS	U	NS
4-Hydroxy-3-methoxy-benzaldehyde	D	NS	NS	NS	NS
Aspartyl-Glycine	D	NS	NS	NS	NS
4-Ethylphenol	D	NS	NS	NS	NS
Glutamyl-Lysine	NS	D	D	NS	D
L-2-Aminoadipate 6-semialdehyde	NS	U	NS	NS	NS
2-Amino-5-oxohexanoate	NS	U	NS	NS	NS
Seryl-Threonine	NS	D	NS	NS	NS
2,4-Diaminobutyric acid	NS	D	D	NS	NS
3-Methoxy-4-hydroxyphenylacetaldehyde	NS	NS	D	NS	NS
gamma-L-Glutamylputrescine	NS	NS	D	NS	NS
Gentisate aldehyde	NS	D	D	NS	D
3-Hydroxymandelic acid	NS	NS	D	NS	NS
o-Cresol	NS	NS	D	NS	NS
Glycyl-Proline	NS	NS	U	NS	NS
Alpha-aminobutyric acid	NS	NS	U	NS	NS
3S,5S-3,5-Diaminohexanoate	NS	NS	U	NS	NS
3,4-Dihydroxy-L-phenylalanine	NS	NS	D	NS	NS
2-Pyrocatechuic acid	NS	NS	U	NS	NS
Sarcosine	D	D	D	D	NS
Vanillylmandelic acid	NS	NS	D	NS	NS
Guanidoacetic acid	NS	NS	NS	U	NS
Cystine	NS	NS	NS	U	NS
R-1-Aminopropan-2-ol	NS	NS	NS	D	NS
Homoarginine	NS	NS	NS	U	NS
Cystathionine - Isomer	NS	NS	NS	D	NS
Homoserine	NS	NS	NS	U	NS
L-Selenocystathionine	NS	NS	NS	U	U
Ornithine	NS	NS	NS	U	NS

trans-2,3-Dihydroxycinnamate	NS	NS	NS	D	NS
4-Aminobutyraldehyde	NS	NS	NS	U	NS
N6-Acetyl-N6-hydroxy-L-lysine	NS	NS	NS	U	NS
Histidine	NS	NS	NS	U	NS
Isomer of 1-Aminocyclopropane-1-carboxylate	NS	NS	NS	U	D
Methylguanidine	NS	NS	NS	U	NS
Aminoadipic acid	NS	NS	NS	NS	D
4-Hydroxybenzoic acid	NS	NS	NS	NS	D
Leucyl-Aspartate	NS	NS	NS	NS	U
2-Hydroxy-2,4-pentadienoate	NS	NS	NS	NS	D
2R,5S-2,5-Diaminohexanoate	U	NS	NS	U	U

(Abbreviations: D=downregulated, U=upregulated, NS=not significant)

Table A1.17: Disease specific metabolites in AD patients in different food groups

Disease specific metabolites	Egg	Milk	Peanut	Sesame	Soy
3,4-Dihydroxybenzeneacetic acid	U	U	U	U	U
5-Acetylamino-6-amino-3-methyluracil	D	NS	D	D	U
Aspartyl-Threonine	U	U	U	NS	U
L-Aspartate 4-semialdehyde	D	NS	D	NS	NS
Leucyl-Aspartate	U	U	U	NS	U
L-Glutamate 5-semialdehyde	U	NS	U	NS	NS
Leucyl-phenylalanine	U	U	U	D	U
Glutamyl-Methionine	D	D	D	D	D
Alanyl-Valine	U	U	U	NS	U
Uridine	U	U	U	NS	U
Theophylline	D	NS	D	NS	NS
Phenylalanyl-Tryptophan	U	NS	U	U	NS
5-Hydroxyindoleacetic acid	D	NS	D	U	NS
L-2-Aminoadipate 6-semialdehyde	U	NS	U	NS	NS
Glycyl-Phenylalanine	U	U	U	NS	U
2'-Deamino-2'-hydroxy-6'-dehydroparomamine	D	D	D	NS	D
Methionine Sulfoxide	NS	D	NS	NS	D
Pipecolic acid	NS	U	NS	NS	U
3-Sulfino-L-alanine	NS	U	NS	NS	U
o-Cresol	NS	U	NS	NS	U
Leucyl-Isoleucine	NS	U	NS	NS	U
4-Ethylphenol	NS	U	NS	NS	U
Gentisic acid	NS	U	NS	NS	U
N6-Acetyl-N6-hydroxy-L-lysine	NS	U	NS	NS	U
Prolyl-Lysine	NS	D	NS	NS	D
2,4-Diaminobutyric acid	NS	U	NS	NS	U
S-Glutathionyl-L-cysteine	NS	U	NS	NS	U
2-Aminooctanoic acid	NS	NS	NS	D	NS
Lysyl-Glutamine	NS	NS	NS	U	NS
Taurine	NS	NS	NS	U	NS
3-Methoxy-4-hydroxyphenylacetaldehyde	NS	NS	NS	U	NS
trans-3-Hydroxy-L-proline/cis-3-Hydroxy-L-proline	NS	NS	NS	D	NS
4-Hydroxy-3-methoxy-benzaldehyde	NS	NS	NS	U	NS
prolyl-proline	D	U	U	U	U

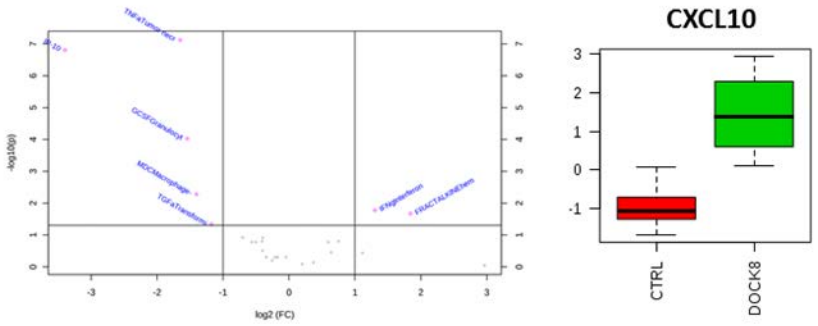
(Abbreviations: D=downregulated, U=upregulated, NS=not significant)

Table A1.18: Most significant pathways in DOCK8-deficient and AD patients

Food groups	DD (p-value<0.05)	AD (p-value<0.05)
Egg	Glycine, serine and threonine metabolism	Caffeine metabolism
	Phenylalanine, tyrosine and tryptophan biosynthesis	
	D-Arginine and D-ornithine metabolism	
	Arginine and proline metabolism	
	Tryptophan metabolism	
Milk	Taurine and hypotaurine metabolism	Lysine degradation
		Cysteine and methionine metabolism
		Tyrosine metabolism
		D-Glutamine and D-glutamate metabolism
Peanut	Lysine degradation	Caffeine metabolism
	Arginine and proline metabolism	
Sesame	Arginine and proline metabolism	Tyrosine metabolism
	beta-Alanine metabolism	Taurine and hypotaurine metabolism
	Tryptophan metabolism	
	Nitrogen metabolism	
	Lysine degradation	
Soy	Phenylalanine metabolism	Lysine degradation
	Selenoamino acid metabolism	Cysteine and methionine metabolism
		Tyrosine metabolism
		D-Glutamine and D-glutamate metabolism

Figures

A



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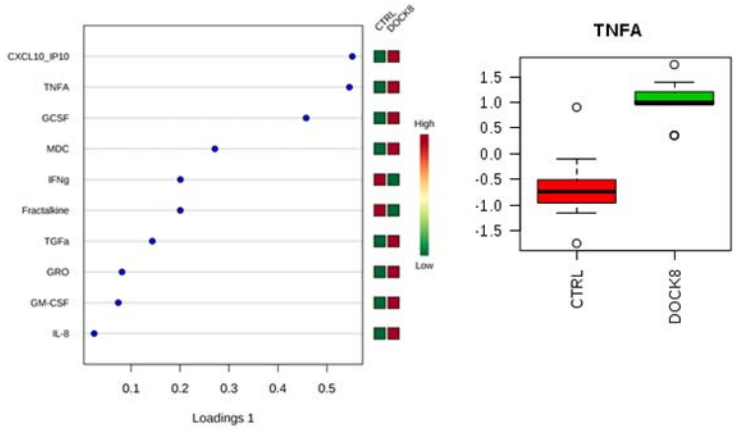

Figure A2.1: Binary comparison between DOCK8 deficient patients and controls (**A**) Volcano plots show up regulation of CXCL10 and TNFA in DOCK8 deficient patients compared to healthy controls. **(B)** Loading plots for DOCK8 vs control.

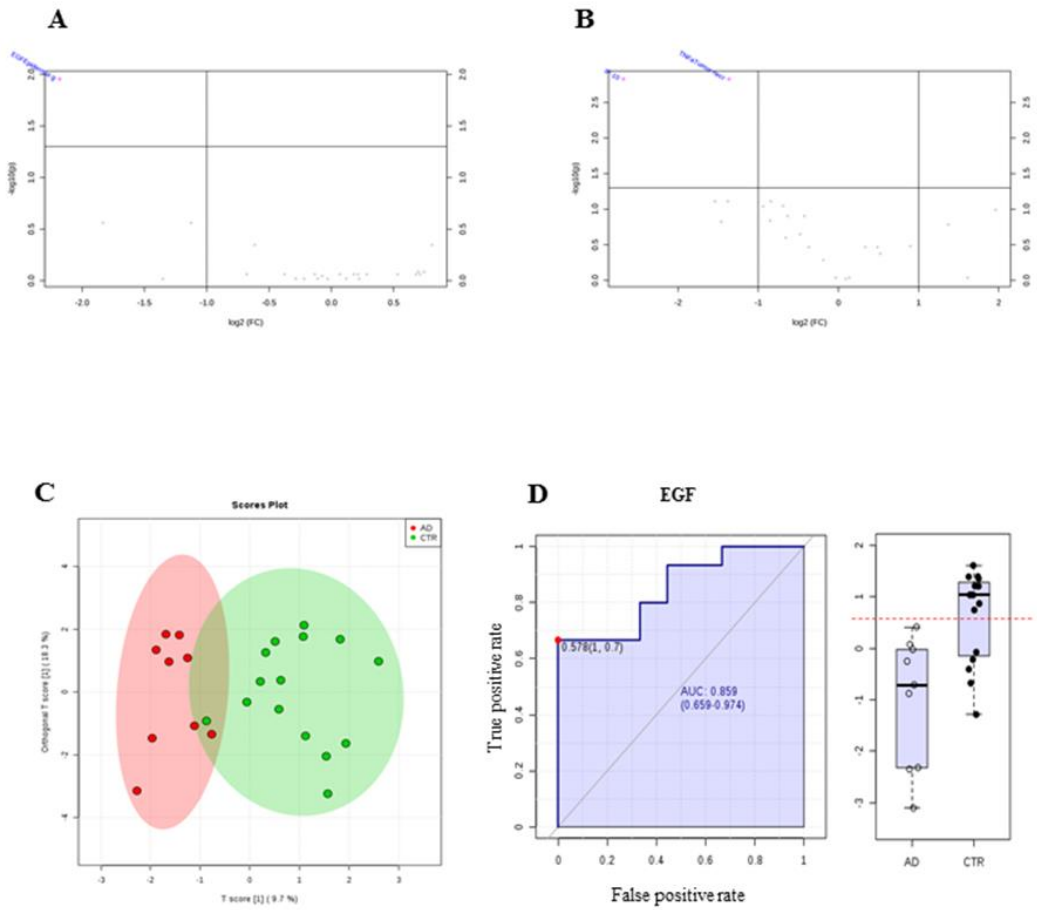

Figure A2.2: Volcano plots and Binary comparison: (**A**) AD vs ctrl,(**B**) DOCK8 vs AD (**C**) Comparison between AD patients and healthy controls (Q2=0.32) where Q2 represents the level of differential expression of the panel analytes (D) Epidermal growth factor (EGF) is down-regulated in the AD patients when compared to healthy ctrls, AUC: 0.859

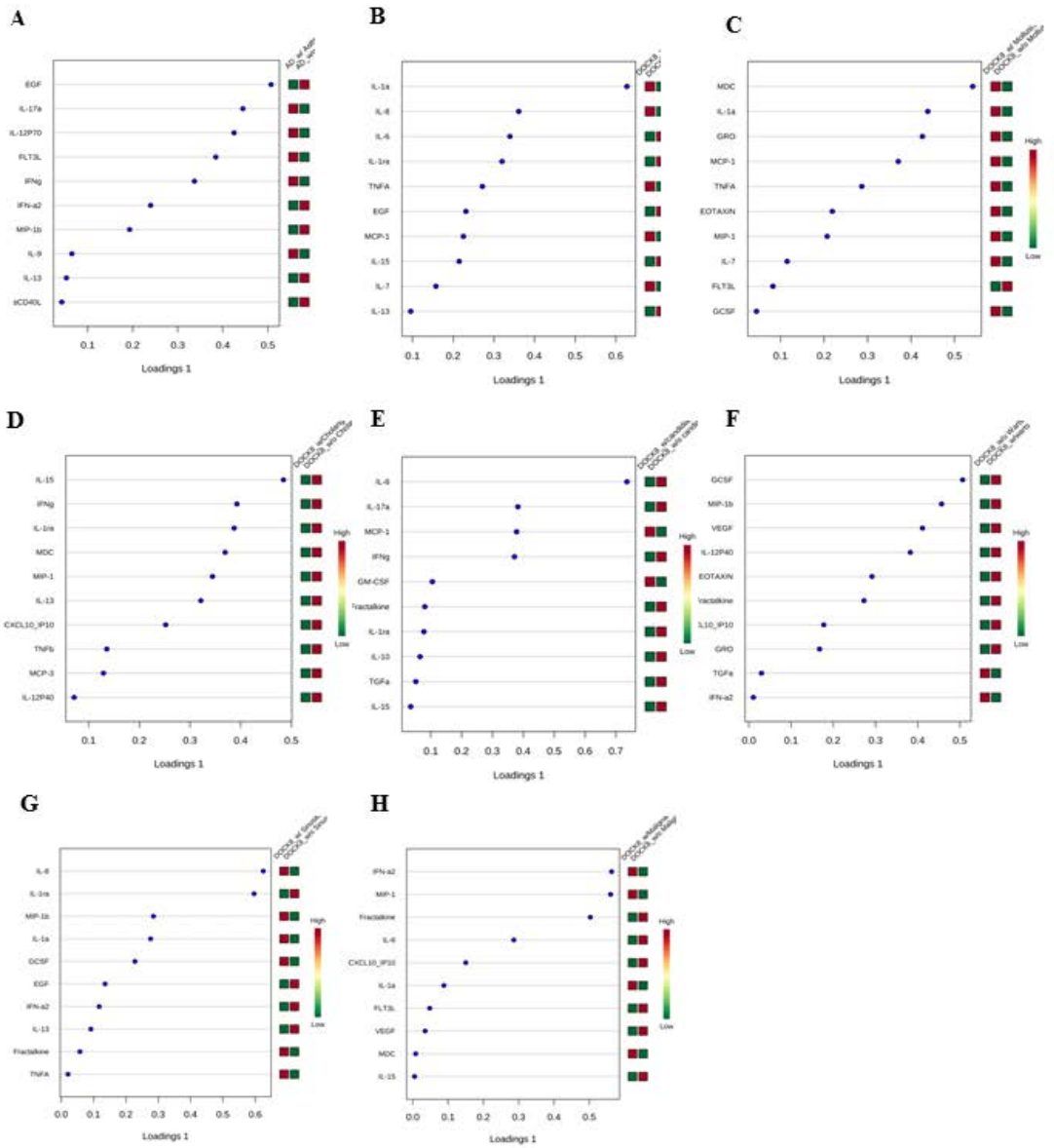

Figure A2.3: PLS-DA loading plots based on binary comparisons in AD and DOCK8 deficient patients with/without various clinical phenotypes including (**A**) asthma, (**B**) bronchiectasis, **(C)** molluscum contagiosum, (**D**) sclerosing cholangitis, (**E)** candidiasis, (**F**) warts, (**G**) sinusitis, (**H**) malignancy.

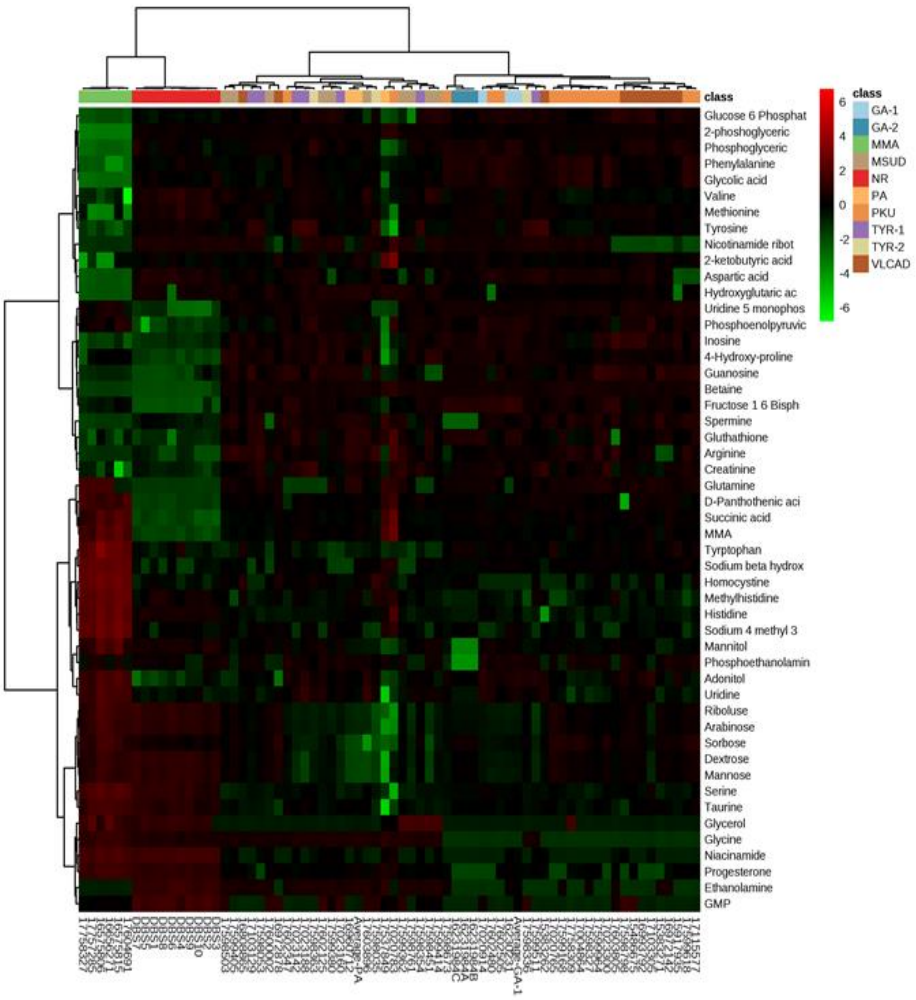

Figure A2 4 : Heat map for all 56 abnormal samples

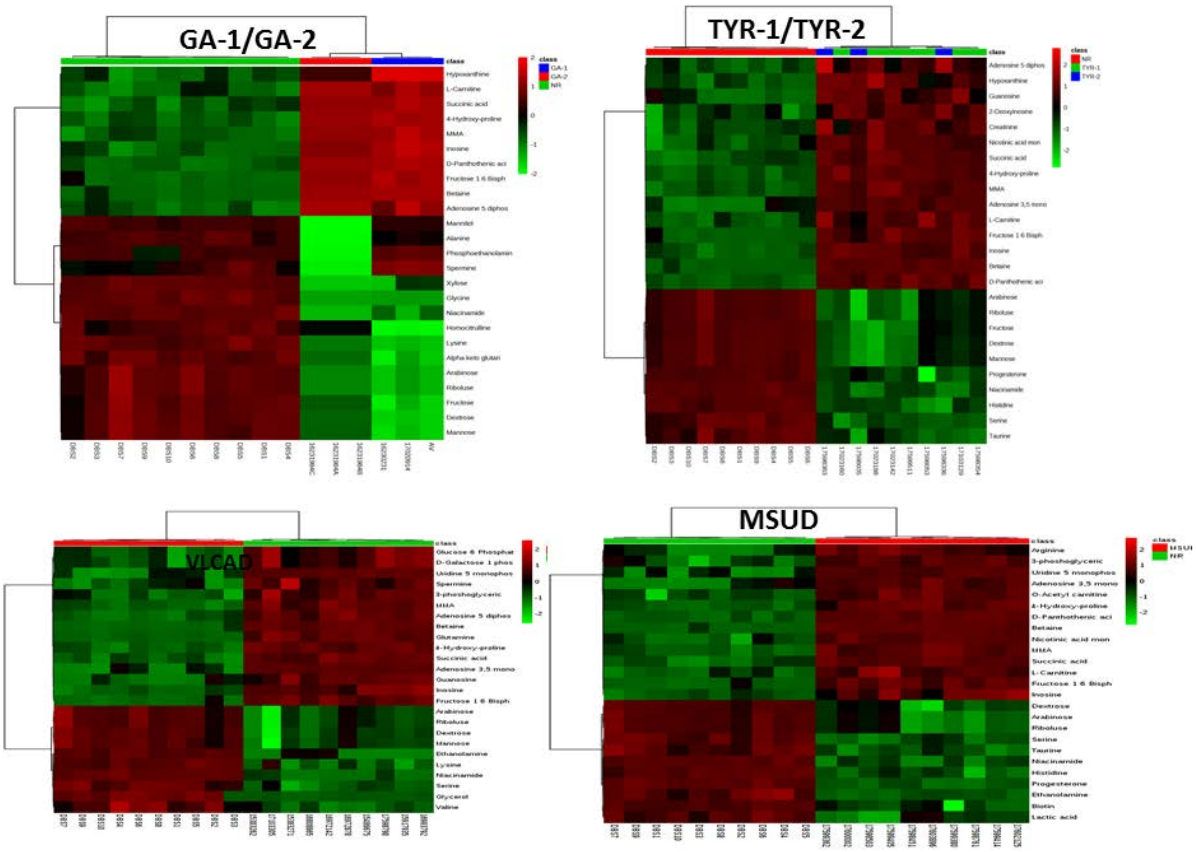

Figure A2.5 : Heat maps for GA-1/2, Tyr1/2, VLCAD and MSUD

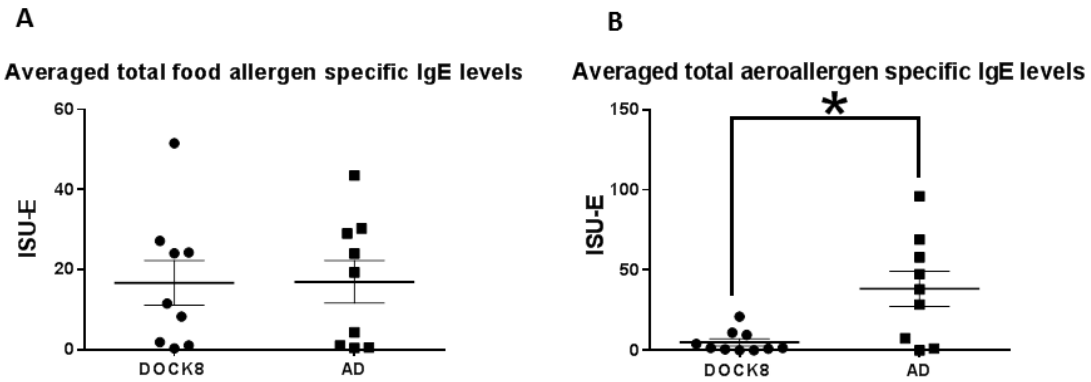

Figure A2 6: ISAC profiling in DOCK8-deficient (n=10) and atopic dermatitis (n=9) patients is significant for aero-allergens but not for food-allergens.(A) Food-allergens (B) Aero-allergens measured in ISU-E units.(* Indicates significance. Not specified ones are not significant, p-value< 0.05,)
Abbreviations: AD-Atopic dermatitis, ISU-E (ISAC Standardized units)

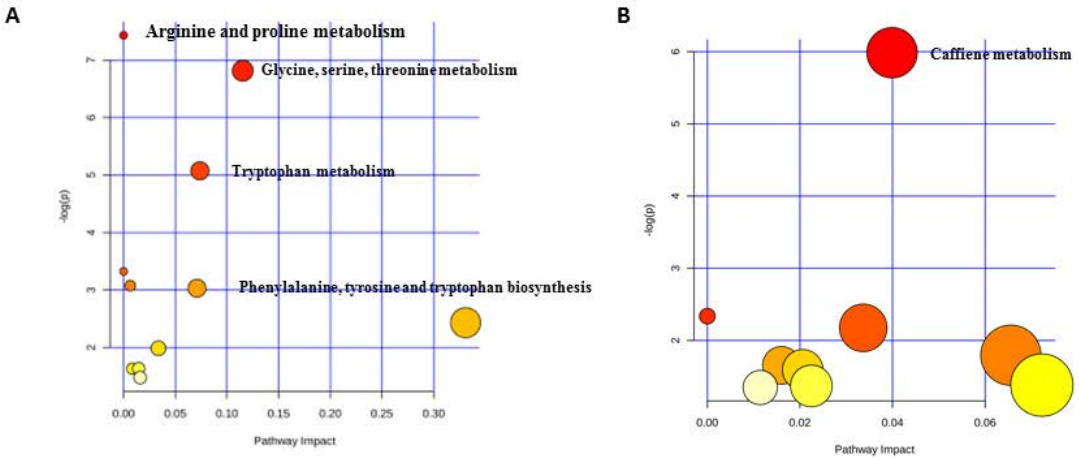

Figure A2 7 : Differential pathway involvement is revealed in (A) DOCK8-deficient patients (n=10) and (B) atopic dermatitis patient (n=9)

APPENDIX B: PUBLICATIONS FROM THIS THESIS

B1.1 Minnie Jacob, Andreas L. Lopata, Majed Dasouki, Anas M. Abdel Rahman- Metabolomics toward personalized medicine. *Mass Spectrom Rev*. 2019.38(3):221-238...

B1.2 Minnie Jacob, Duaa Bin Khalaf, Safa Alhissi, Rand Arnout, Bander Alsaud, Hamoud Al-Mousa Andreas L. Lopata, Anas M. Alazami, Majed Dasouki, Anas M. Abdel Rahman- Quantitative profiling of cytokines and chemokines in DOCK8 deficient and Atopic dermatitis patients . *Allergy*. 2019;74(2):370-379

B1.3 Minnie Jacob, Abeer Malkawi, Nour Albast, Salam Al Bougha, Andreas Lopata, Majed Dasouki, Anas M. Abdel Rahman. A targeted metabolomics approach for clinical diagnosis of Inborn Errors of Metabolism. *Anal Chim Acta*. 2018; 1025: 141-153.

B1.4 Minnie Jacob, Xian Luo, Xian Luo, Rand Arnout, Bander Alsaud, Hamoud Al-Mousa Andreas L. Lopata, Liang Li, Majed Dasouki, Anas M. Abdel Rahman- Metabolomics distinguishes DOCK8 deficiency from atopic dermatitis:Towards a biomarker discovery. *Metabolites.*2019 Nov 12, 9 (11), doi: 10.3390/metabo9110274

APPENDIX C: PUBLICATIONS DRAFTED/ SUBMITTED

C1.1 Minnie Jacob, Xian Luo, Rand Arnout, Bander Alsaud, Hamoud Al-Mousa, Liang Li,Majed Dasouki,Andreas L Lopata ,Anas M. Abdel Rahman: Distinct IgE based allergen array, metabolomics and cytokine profiles in DOCK8-deficient and atopic dermatitis patients.*Allergy* (drafted)